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MUTATIONS IN PATHOGENICITY OF SOME RECENTLY ISOLATED STRAINS OF FOWL-PLAGUE VIRUS

By

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INTRODUCTION

In a footnote to a recent paper, Daubney, Mansi and Zahran (1949) briefly reported that one recently isolated strain of fowl-plague virus had yielded a mutant, apathogenic for domestic fowls, pigeons, and turkeys, and highly antigenic for fowls. The present paper is largely concerned with the behaviour of this particular strain, but it also records attempts to isolate mutants from three other freshly isolated field strains.

MATERIALS AND METHODS

Strains of Fowl-Plague Virus. Recently isolated strains used in these experiments are described in the text. The strain used for challenging birds is the original "A" strain, referred to in previous papers. It has been maintained by serial passage in chick-embryos, and its full virulence has from time to time been restored by a few passages in susceptible fowls.

Experimental Birds. Most of the susceptible birds have been young unvaccinated Fayoumi or cross-bred European birds from the farms of the Ministry of Agriculture. Occasionally, when such birds have not been obtainable, it has been necessary to buy young birds elsewhere. Such birds have been vaccinated with fowl-pox vaccine and held for a few days under observation before being placed on experiment. Most of the birds, including all from outside sources, have been subjected to a preliminary haemagglutination-inhibition test as a measure of their susceptibility to the viruses of Newcastle disease and fowl plague.

Embryos. Hen's eggs, largely from cross-bred flocks immunized against Newcastle disease and fowl plague have been obtained from the Ministry's Poultry Farms and from outside breeders. They have been inoculated on the 10th day by the intra-allantoic route. Pigeon embryos have been obtained from various lofts, because the pigeon loft at the Institute has never provided a sufficient number. They have been inoculated at an estimated age of 7 to 9 days by the intra-allantoic route.

Haemagglutination CCA and Haemagglutination-inhibition Tests. The haemagglutination test has been used to check the virus content of passage embryos, employing a 0.75 per cent suspension of chick or horse cells. When haemagglutination-inhibition tests have been employed as an index of immunity, the first post-vaccination check test has generally been carried out on the 12th day after inoculation. Subsequent tests have been made on those birds in which the response by the twelfth day has appeared to be partial only. The rough titres given indicate the maximum dilution of serum which gave complete inhibition in a series of twofold dilutions with 1 to 2 units of antigen. Birds which after exposure to living virus, inhibit in a dilution of 1 : 160 and over are invariably found to be

immune. Almost all birds with a titre of 1 : 80 will equally be found to be immune, but an occasional one will succumb on challenge. Of birds that show titres lower than 1 : 80 a few will prove on challenge to be immune.

Desiccations. Desiccated preparations—generally consisting of whole egg content, with or without the albumen yolk and yolk sac—are prepared by grinding in an Atomix blender, wet-dispensing in 0.5 cc or 1 cc amounts in 11 mm.o.d. ampoules, and spin-freeze drying in an Edwards Model No. 3 desiccator. Primary desiccation is continued for 20 hours with little or no additional heat, and secondary desiccation over $P_2 O_5$ is carried out for a further period of 16 to 20 hours. Sealed ampoules are stored at −20° C. Residual moisture content is below 0.5 per cent.

EXPERIMENTAL

After a number of fruitless exploratory trials with established laboratory strains of fowl-plague virus, which were mentioned in an earlier paper, three recently isolated strains were selected for further study in the hope that an avirulent mutant might be isolated. Later a fourth freshly isolated strain was subjected to preliminary examination.

Strains 319 *and F.A.*

The first of these strains was Strain 319, isolated in February, 1948, from a sick bird purchased in Fayoum market at a time when an epizootic of Newcastle disease was prevailing in this province. Altogether 12 sick birds were bought on a single day in the market, and from these birds 7 strains of Newcastle-disease virus, and one of fowl plague were recovered. Strain 319 killed chick embryos rather more slowly than is normal with recently isolated fowl-plague strains, the incubation period in inoculated birds was longer than normal, and the pattern of haemagglutination by the virus was intermediate between that of Newcastle disease and that of fowl plague. Strain 319 is considered to resemble the atypical strains described by Lagrange (1929) and later investigated by Purchase (1931).

The second strain was Strain F.A. which was isolated from an outbreak of classical fowl-plague of the acute type in adult turkeys on the Experimental Farm of the Faculty of Agriculture, Fouad 1st University, Cairo, also in February, 1948.

Stains 319 and F.A. were passaged serially in duck embryos for 51 and 52 passages, and in pigeon embryos for 87 and 46 passages, respectively, without the occurrence of any significant change in the virulence of the strains for fowls as Table I will show.

The pigeon-embryo and duck-embryo series of Strain 319 were started from the 5th chick-embryo passage. The usual survival period of the embryo was 42 hours with this strain; this applies equally to pigeon, duck or chick embryos. From the 72nd pigeon-embryo passage of Strain 319, when it seemed just possible that some mutation might be occurring, two separate lines were maintained,

TABLE I

STRAIN 319 IN PIGEON AND DUCK EMBRYOS

Passage	*No. inoculated*	*Deaths after inoculation*	*Serum inhibition titre*	*Survival rate on challenge*
3rd Chick embryo	4 fowls	*D4, D4, D6, D6	—	—
51st Duck embryo	4 fowls	D4, D4, D4, D4	—	—
13th Pigeon embryo	6 fowls	D3,D3,D4,D4,D6,S.	80 (1)	1/1
36th Pigeon embryo	6 fowls	D4,D5,D6,D7,D8,S.	—	—
63rd Pigeon embryo	3 fowls	D5, D10	10 (1)	Not challenged
72nd Pigeon embryo	6 fowls	D7,D7,D8,S.S.S.	320 (2)	2/2
87th Pigeon embryo (a)	9 chicks 2½ months	D3,D4,D4,D4,D4 D5,D5,D5,D7	—	—
87th Pigeon embryo (b)	9 chicks	D3,D3,D5,D5,D5 D5,D5,D5,D6	—	—
		STRAIN F.A. IN PIGEON AND DUCK EMBRYOS		
1st Chick embryo	3 fowls	D2, D3, D3	—	—
52nd Duck embryo	4 fowls	D4,D5,D5,D5	—	—
36th Pigeon embryo	5 fowls	D2,D3,D4,D4,S.	80 (1)	1/1
46th Pigeon embryo	4 fowls	D2,D4,D4,D5	—	—

*D4 indicates one death on the 4th day.
S.=survived.

one inoculated with a 10^{-1} dilution of allantoic fluids of the preceding passage, and the other with a 10^{-6} dilution; they are termed 'a' and 'b' respectively in Table 1.

Strain P.F.

The third strain selected for investigation was Strain P.F., isolated in April, 1948, from freshly imported Indian pea-fowl at the Zoological Gardens, Cairo. The birds travelled by air from Calcutta, and deaths from fowl plague occurred among the group within 24 hours of arrival. The strain was highly virulent for the pea-fowl, and none of the group survived beyond the first week. Strain P.F. was if anything slightly below normal virulence for fowls inoculated with 1 cc x 10^{-3}; of embryonic fluids, 8 of the birds dying on the 4th or 5th days. At the 8th chick-embryo passage it also killed 10/10 inoculated birds, 9 of them dying on the 4th and 5th days.

This strain was transferred to pigeon embryos after 2 chick-embryo passages. The survival period of inoculated pigeon embryos in the early passages was irregular, but rather longer than with strains F.A. and 319, ranging from 2 to 6 days. From the 11th passage onwards it shortened to 2 to 3 days, and eventually in later passages 2 days became the normal, with younger embryos frequently dying in 20 hours.

At the 11th pigeon-embryo passage the pooled fluids from 2 pigeon embryos were roughly titrated in chick embryos, and 5 fowls

were inoculated with 1 cc x 10^{-3} of the fluids. The chick-embryo titration extended only from $10^{-3 \cdot 7}$ to $10^{-6 \cdot 7}$, with 3 embryos per dilution, but all embryos died and all fluids showed high CCA titres. All 5 inoculated birds survived without symptoms, and none showed haemagglutination inhibition by the 14th day. All 5 died on challenge in 2 to 4 days. Because of the survival of these 5 birds, five additional birds Nos. 466-470 were inoculated with 1 cc x 10^{-2} of stored fluids from the 11th pigeon-embryo passage (PFC_2 P_{11}), and 5 others Nos. 471-475 with a suspension of ground chorio-allantoic membrane of PFC_2 P_{13}. These inoculations were performed before the challenge of the first group of 5; but when the first group proved to be susceptible the strain at the 11th passage was returned to chick embryos. After 2 passages in chick embryos (PFC_2 P_{11} C_2), 1 cc of a 20 per cent suspension of embryonic fluids was given as a second injection to 5 of the 10 birds that had already been inoculated 8 days earlier with no apparent effect. This second treatment was given to 2 birds of the first (PFC_2 P_{11}) group, and 3 birds of the second group (PFC_2 P_{13}). The results of challenging these birds with virus are tabulated, (Table II).

TABLE II

	1st Inoculation	*2nd Inoculation*	*Number Inoculated*	*Survival rate on challenge 5/8/48*
	PFC_2 P_{11} fluids	—	3 Fowls	3/3
Group I 5 Fowls	CE titre 10^{-6} 15/7/48	PFC_2 P_{11} C_2 fluids 23/7/48	2 Fowls	2/2
	PFC_2 P_{13} membrane	—	2 Fowls	0/2 D3, D7
Group II 5 Fowls	15/7/48	PFC_2 P_{11} C_2 fluids 23/7/48	3 Fowls	3/3
	Susceptible controls	—	3 Fowls	0/3 D3,D3,D3

CE=chick embryo.

This small trial indicated that after 11 pigeon-embryo passages the PF strain was no longer lethal to fowls, and in certain circumstances was capable of immunizing them solidly against challenge with virulent fowl-plague virus. Apparently the 13th pigeon-embryo passage material had lost the power to evoke immunity, and the immunity of the three birds in group II that resisted challenge is to be attributed to the second inoculation of PFC_2 P_{11} C_2.

The direct pigeon-embryo line was carried to the 24th passage (PFC_2 P_{24}). At the 16th pigeon-embryo passage a transfer was made

in chick embryos to obtain material for the inoculation of fowls, and birds were again inoculated at the 24th pigeon-embryo passage. It is clear from the tabulated results (Table III) that the capacity to establish an infection—and so to immunize fowls—was not fully retained in the direct pigeon-embryo passaged line beyond the 11th pigeon-embryo passage, although 3 birds out of 10 were protected by material of 16th pigeon-embryo passage.

TABLE III

Serial Passage	*Material and Dosage*	*Number inoculated*	*Result of inoculation*	*Survival rate on challenge*	*Remarks*
$PFC_2\ P_{16}\ C_1$	1cc x 10^{-2} AAF	10 Fowls	—	3/10	$D_2,D_3,D_3,D_4,D_5,D_5,D_5$.
$PFC_2\ P_{24}$	1cc x 10^{-1} AAF	5 Fowls	—	0/5	D_3,D_4,D_4,D_5,D_7.
	1cc x 10^{-2} AAF	5 Fowls	—	0/5	D_3,D_3,D_4,D_4,D_5.

AAF=allantoic and amniotic fluids

Repassage of $PFC_2\ P_{11}\ C_2$ was continued in chick embryos also. In the chick-embryo repassaged strain (Table IV) originating with $PFC_2\ P_{11}\ C_2$ the capacity regularly to infect fowls was not in evidence at the 3rd, 5th, 7th and 26th chick-embryo passages, although it had been evident in the 2nd chick-embryo repassage (*vide* Table II). There is, however, in both the pigeon-embryo and chick-embryo series a sugestion of some faint antigenic effect in that a few birds resisted challenge completely, and the survival period of others was longer than usual. There had been no loss of CCA activity during these passages in either the pigeon or chick-embryo lines, infectivity titres in chick embryos of both series had remained satisfactory, and the survival time of chick embryos had been 40 to 42 hours throughout.

Since the consistently negative results of inoculating fowls with the chick-embryo repassaged material up to the 26th repassage contrasted strongly with the substantial C.C.A. and chick-embryo infectivity titres of the material it was decided to find out (a) whether a batch of inactivated adjuvant vaccine prepared according to our routine method from the re-passaged virus would protect birds, and (b) if the addition of adjuvant substances would raise the attacking power of the strain. At the 31st chick-embryo repassage an oily-based vaccine was prepared from the whole egg content by formalisation and suspension of the material in lanolin and P 40 oil containing autoclaved mammalian tubercle bacilli, in the manner described by Daubney, Mansi and Zahran (1949). A similar emulsion was made with active materials from the same pool of eggs; and yet another portion of the material was treated by the

addition of 50 per cent aluminium gel. Three fowls were inoculated intramuscularly with 1 cc, and 3 with 0·5 cc, of the inactivated oily-based vaccine. Four birds were inoculated with the active oily-based preparation in 1 cc doses, and 4 with 1 cc each of the aluminium gel preparation. None of the birds showed any reaction, and all completely resisted challenge on the 14th day. Since the four birds that had been inoculated with 1 cc$\times 10^3$; doses of the untreated allantoic fluids of the 31st repassage also resisted challenge at the same time (Table IV) and had already shown high H.I. titres, the trial with the active adjuvant-treated materials had little point, beyond increasing from 4 to 18 the number of birds successfully immunized with Passage 31 material.

TABLE IV

Serial passage	*Material and and dosage*	*Number inoculated*	*Result of inoculation*	*Survival rate on challenge*	*Remarks*
$PFC_2\ P_{11}\ C_3$	1cc x 10^{-2} AAF	10 Fowls	—	1/10	D_2,D_4,D_5,D_5, D_5,D_5,D_5,D_6, D_6.
$PFC_2\ P_{11}\ C_5$	1cc x 10^{-2} AAF	8 Turkeys	—	2/8	D_2,D_2,D_2,D_3, D_3,D_5.
		10 Fowls	—	1/10	D_2,D_3,D_3,D_3, D_3,D_3,D_4,D_7, D_{10}.
$PFC_2\ P_{11}\ C_7$	1cc x 10^{-2} AAF	10 Fowls	—	0/4	D_4,D_4,D_5,D_5.
$PFC_2\ P_{11}\ C_{26}$	1cc x 10^{-3} AAF	3 Fowls	$<1:10<1:10<1:10$	0/2	D_7,D_7.
Susceptible controls		6 Fowls	—	0/6	D_3,D_3,D_4,D_4, D_4.D_5.

The capacity to attack fowls regularly and to evoke immunity was eventually regained between the 26th and 30th chick-embryo repassages. Trials with the chick-embryo repassaged line, once the capacity to attack fowls had been restored, gave rather erratic results. In a titration of $PFC_2\ P_{11}\ C_{34}$ in which 6 groups, each of 3 adult birds, were inoculated with tenfold dilutions of desiccated whole egg content, ranging from 1 cc x 10^{-3} to 1 cc x 10^{-8}, two only of the 3 birds inoculated with the lowest dilution showed CCA inhibition after the inoculation. All 3 birds inoculated with this dilution resisted subsequent challenge with virulent fowl-plague virus. The sera of the remaining 15 birds showed no inhibition, and they were not subjected to challenge. It is of interest to note that a simultaneous titration of this dried material in chick embryos, covering the range 10^{-3} to 10^{-10}, in tenfold steps with 5 embryos at each dilution, gave 5/5 deaths at 1 cc x 10^{-3}, 10^{-4}, and 10^{-5}, and 2/5 deaths at 10^{-6}, all confirmed by CCA tests of the embryonic fluids.

In a similar test of the infectivity of fresh embryonic fluids of PFC_2 P_{11} C_{33}, fowl and chick-embryo titrations gave the following results :—

Fowls. 10^{-3}—2/2 10^{-4}—2/2 10^{-5}—0/1 10^{-6}—1/2 10^{-7}—0/2 10^{-8}—0/2; Chick Embryos. 10^{-6}—5/5 10^{-7}—5/5 10^{-8}—5/5 10^{-9}—3/5.

In the case of the fowl titration the fractions represent survivors over challenged. One bird inoculated with 1 cc x 10^{-3} was noticeably sick on the 5th and 6th days after inoculation with the mutant or "attenuated" virus, and one of the 10^{-5} pair died on the 8th day, apparently from the effect of the virus, which was recovered from the brain.

A small experiment to demonstrate interference by the mutated strain was carried out with embryonic fluids from the titration embryos of PFC_2 P_{11} C_{33}. Table V gives the result of this trial, and it will be seen that a dose of 1 cc x 10^{-3} of fluids quite definitely interfered with a dose of 1 cc x 10^{-5} of a virulent strain given 2, 3, or 5 days after the first inoculation.

TABLE V

Strain	*Dose*	*Challenge*	*Interval before challenge*	*Survivors*	*Remarks*
			—1 day	0/3	D4, D5, D6
			—2 days	1/3	D6, D7. Remaining bird sick 2 days
PFC_2 $P_{11}C_{34}$	1 cc x 10^{-3} AAF	1 cc x $10^{-5.72}$ strain "A"	—3 days	3/3	One bird sick 6th—8th days.
			—		
			—		
			—5 days	2/3	D8. Death may have been due to PFC_2 P_{11} C_{34}, and not to challenge.
			—		
			—		
			—7 days	3/3	—

The fact that one bird inoculated with PFC_2 P_{11} C_{33} had apparently been killed by the virus, and that others had been made ill, was taken as an indication that continued repassage of this line in chick embryos would probably lead to a restoration of its virulence for fowls. Consequently, the strain was returned to pigeon embryos at the 30th and 34th chick-embryo repassages for further serial passage, and at the same time fresh lines were started in pigeon embryos from stored material of PFC_3 and PFC_2 P_6.

PFC_2 P_{11} C_{30} P . . . The behaviour of the virus in the series originating with PFC_2 P_{11} C_{30} is summarized in Table VI. It will be observed that virus of the 1st pigeon-embryo repassage showed

TABLE VI

$PFC_2\ P_{11}\ C_{30}\ P_1$

Serial passage	*Material and Dosage*	*Number inoculated*	*Result of inoculation*	*Serum inhibition titres*	*Survival rate on challenge*
$PFC_2\ P_{11}\ C_{30}\ P_1$	AAF 10^{-3}	8 Fowls	D9,D10,D10,D11		4/4
$PFC_2\ P_{11}\ C_{30}\ P_{18}$	AAF 10^{-3}	8 Fowls	D16	320(3) 20(1) 0(3)	7/7
$PFC_2\ P_{11}\ C_{30}\ P_{30}$	AAF 10^{-3}	6 Fowls	—	80(3) 40(2) 0(1)	4/5
$PFC_2\ P_{11}\ C_{30}\ P_{40}$	AAF 10^{-3}	8 Chicks 2 months	D3,D8,D10	320(1) 160(1) 80(1) 40(2)	4/5
$PFC_2\ P_{11}\ C_{30}\ P_{59}$ Whole egg content	10^{-3}	8 Chicks $4\frac{1}{2}$ months	—	320(7) 0(1)	3/5
$PFC_2\ P_{11}\ C_{30}\ P_{80}$	10^{-3}	3 Chicks $2\frac{1}{2}$ months	—	320(2) 80(1)	2/2
Whole egg content	10^{-5}	3 Chicks $2\frac{1}{2}$ months	—	340(1) 20(1) <20(1)	1/3
	10^{-7}	3 Chicks $2\frac{1}{2}$ months	—	<20(3)	0/3
$PFC_2\ P_{11}\ C_{30}\ P_{102}$	AAF 10^{-3}	20 Fowls	D5. virus +	320(17) <20(2)	
$C_{30}\ P_{120}$	AAF 10^{-3}	20 Fowls	D7. virus +	320(16) 160(2) 80(1)	

unexpected pathogenicity in that it killed 4 fowls out of 8, immunizing the remaining 4. After 18 pigeon-embryo passages pathogenicity had declined, since 7 birds out of 8 survived and were immunized, and the single death did not occur until the 16th day after inoculation. At the 40th repassage 3 of 8 chicks that were inoculated died. This was an unusually weak batch of chicks in which mortality was heavier than normal irrespective of the strain with which they were inoculated. In tests of the 59th and 80th repassages there was no mortality among chicks inoculated, and all except one of the 11 birds that received 1 cc x 10^{-3} were apparently immunized. Unfortunately it was not possible to challenge the birds inoculated with the 59th repassage.

At the 85th repassage a quantity of whole egg-content from a pool of eggs was freeze-dried and titrated in decimal dilutions in chick-embryos and 4 month-old birds. The LD 50 of the freeze-dried preparation for chick-embryos was 1 cc x 10^{-8}; in terms of

TABLE VII

PFC_2 P_{11} C_{34} RETURNED TO PIGEON EMBRYO

Serial passage	*Material and Dosage*	*Number inoculated*	*Result of inoculation*	*Serum inhibition titres*	*Survival rate on challenge*	*Remarks*
C_{34} P_1	AAF 10^{-2}	8 Fowls	D10	320(1) 160(3) 40(1) 0(2)	3/6	0 40 0 D_3, D_3, D_5.
C_{34} P_{17}	AAF 10^{-2}	8 Fowls	D7,D17	320 (3) 160(1) 80(1) 0(1)	6/6	—
C_{34} P_4	AAF 10^{-2}	8 Chicks (2 months)	D2,D3,D3,D4, D5, D6, D7	—	—	—
C_{34} P_5	Whole egg content 10^{-3}	8 Chicks $4\frac{1}{2}$ months)	—	320(6) 160(1) 20(1)		
C_{34} P_{80}*	Whole egg content 10^{-3}	3 Chicks (3-4 months)	D6,D7,D9	—	—	—
	Whole egg content 10^{-5}	3 Chicks	D6,D10,D13	80(1)	—	—
	Whole egg content 10^{-7}	3 Chicks	D7	80(2)	1/2	D2

*In this trial virus (PFC_2 P_{11} C_{34} P_{80}), spread by contact to uninoculated controls, killing 4 birds out of 10.

TABLE VIII

B STRAIN OF PFC_2 P_{11} C_{34} P . . .

Serial passage	*Material and Dosage*	*Number inoculated*	*Result of inoculation*	*Serum inhibition titres*	*Survival rate on challenge*	*Remarks*
(B)P_{15} C_1 AAF	10^{-3}	30 Fowls	D15 No Virus	320 (16 160 (10) 80 (1) 40 (1)		Not challenged
(B)P_{15} C_6 AAF	10^{-3}	8 Chicks	D8 Virus +	80 (2) 0 (5)	4/6	0 0 D_3 D12
(B)P_{15} C_6 P_3 AAF	10^{-3}	8 Chicks $2\frac{1}{2}$ months	—	320 (2) 160 (2) 80 (1) 40 (2) <40 (1)	7/8	D6 <40
(B)P_{15} C_6 P_7 AAF	10^{-3}	8 Chicks	D12	320 (4) 80 (2) <40 (1)	6/6	
(B)P_{15} C_6 P_{24} whole egg; less albumen	10^{-3}	8 Chicks $4\frac{1}{2}$ months	—	320 (6) 160 (1) 80 (1)		
	10^{-3}	16 Fowls	—	320 (7) 160 (7) 80 (2)	15/16	1 bird (160) died 20 hours after challenge. No virus. 1 bird (80) slightly sick 6th to 9th days.
(B)P_{15} C_6 P80 AAF	10^{-3}	20 Fowls 7-8 months	D11 No Virus	320 (9) 160 (5) 80 (2)		
			partial	320 (3)		
Susceptible controls	—	4 Fowls	—	—	0/4	D2,D2,D3,G3.

fresh material, and the result in the birds was as follows :—1 ccx10^{-3}, 5/5; 10^{-4}, 2/3; 10^{-5}, 1/3; 10^{-6}, 0/3; 10^{-7} 0/3; 10^{-8}, 0/3. In the 10^{-3} group six birds were inoculated but one died on the 8th day, possibly from the effect of the inoculation although virus could not be recovered from the brain. Six pigeons and six squabs inoculated with 1 cc x 10^{-3} showed no ill effects, and 15 days after inoculation none of their sera showed any power to inhibit CCA. Neither the mortality rate nor the immunization rate appears to have undergone any change during the next 35 passages in pigeon embryos.

PFC_2 P_{11} C_{34} P . . . . The history of PFC_2 P_{11} C_{34} P is a somewhat chequered one, c.f. Table VII. At the first pigeon-embryo repassage 1 bird out of 8 died after inoculation, and 3 only out of 6 challenged proved to be immune. This failure to immunize is doubtless due to low titre and poor adaptation to the pigeon embryo. After 17 pigeon-embryo passages 2 fowls out of 8 died, and the remaining 6 were immunized. The behaviour of the virus in later passages of the series was erratic, but it is clear that at the 80th passage the strain was still quite virulent and the infection capable of spreading by contact.

At the 15th repassage, material of this series was accidentally transferred to chick-embryos together with material of a fairly virulent strain of Newcastle disease virus that was being passaged at the same time by another worker. The accident was not discovered until the mixture of viruses had been passaged 4 times in chick embryos. Separation of the two viruses was readily effected by treating the mixture with fowl-plague or Newcastle-disease immune serum and inoculating the over-neutralized mixture into chick-embryos, as well as by the inoculation of birds immunized against one or the other disease. The recovered (B) PFC_2 P_{11} C_{34} P_{15} strain, which is termed the ' B ' line, proved to be strongly antigenic for fowls and apparently of lower virulence than the parallel line of the same series, (Table VIII). Out of upwards of 140 birds immunized with material from the next 43 pigeon-embryo passages, 3 only have died from the effects of the virus. At (B) P_{15} C_6 P_{25} the chick-embryo titre of desiccated whole egg content was $LD_{50} = 10^{-8}$.

This dried material immunized 6/6 birds at 1 ccx10^{-3}; 2/3 at 10^{-4}; 2/3 at 10^{-5}; 2/3 at 10^{-6}; 1/3 at 10^{-7}; 1/3 at 10^{-8}; 0/3 at 10^{-9}. After 120 days storage at -20°C the same desiccated preparation immunized 15 out of 16 birds inoculated with 1 ccx10^{-3} (vide Table VIII).

Eighteen passages later the titre of a small pool of whole egg content (pigeon) was $LD_{50} = 10^{-7 \cdot 8}$ in the fresh state, which suggests that there may have been a slight fall in titre. After freeze-drying this P43 material immunized 3/3 chicks at 1 ccx10^{-3}; 2/3 at 10^{-5}; 1/3 at 10^{-7}. At P 80 allantoic and amniotic fluids in a dose of 1 cc x 10^{-3} gave a somewhat poorer result, as judged by CCA inhibition titres, which incidentally were slow in developing. Nevertheless

TABLE IX

PFC_2 P_6 STORED BEFORE REPASSAGE

Serial Passage	*Material and Dosage*	*Number*	*Results of inoculation*	*Serum inhibition titres*	*Survival rate on challenge*
PFC_2 P_6 C_1	AAF 10^{-3}	4 Fowls	D3, D3, D4, D4.		
PFC_2 P_6 C_1 P_{16}	AAF 10^{-3}	4 Chicks (3 months)	D6.	20(2) 0(1)	
PFC_2 P_6 C_1 P_{21}	C.E. 10^{-2}	6 Fowls	—	320(1) 160(2) 80(2) 40(1)	6/6
	C.E. 10^{-3}	3 Fowls		320(1) 160(2)	3/3
	C.E. 10^{-4}	3 Fowls	D4, D7.	320(1)	1/1
PFC_2 P_6 C_1 P_{27}	C.E. 10^{-3}	3 Fowls	D3, D7.	160(1)	1/1
	C.E. 10^{-4}	4 Fowls	—	40(1)	3/3
PFC_2 P_6 C_1 P_{30}	AAF 10^{-3}	8 Fowls	D8.	320(2) 160(1) 80(2) 40(2)	7/7
PFC_2 P_6 C_1 P_{37}	AAF 10^{-3}	8 Fowls	D10, D10.	320(6)	6/6
PFC_2 P_6 C_1 P_{71}	AAF 10^{-3}	7 Chicks ($2\frac{1}{2}$ months)	D6, D6.	640(3) 160(1) 0(1)	4/4
PFC_2 P_6 C_1 P_{85}	whole egg content 10^{-3}	8 Chicks' ($4\frac{1}{2}$ months)	D8.	320(7)	—
PFC_2 P_6 C_1 P_{125}	AAF 10^{-3}	10 birds (6-8 months)	D10 fp virus +	320(8) 160(1)	

this is regarded as possibly the best strain so far developed and at this stage it may be considered to have reached a level comparable with that of the Muktesar strain of Newcastle disease vaccine as far as its pathogenicity for birds is concerned, and provided an adequate dose of whole embryo is employed it is likely to be an excellent immunizing agent.

PFC$_2$ *P*$_6$ *C*$_1$ *P* . . . . This series, restarted from stored material of the original 6th pigeon-embryo passage, was at the outset of full virulence *vide* Table IX. There was a fairly rapid decline in virulence during the first 21 passages of the series, but the virus continued to kill a proportion of inoculated birds up to the 85th passage. Of 42 birds inoculated with material from the last 60 passages, excluding the 7 chicks of the highly susceptible group, 8 died, a mortality of approximately 19 per cent. The strain has retained a high capacity to attack fowls and has never failed to immunize the surviving birds. At its present level (125th passage) it is still too virulent, but retains its high antigenic power.

PFC$_3$ *P* . . . . This series was derived from stored material of the second chick-embryo passage. Its virulence for fowls had fallen a little during 86 passages in pigeon embryos. (Table X). Of 36 birds inoculated with material of the last 66 passages, again excluding the one batch of weak chicks, 19, or 52 per cent, died. Of the remainder only one was found on challenge to be susceptible.

TABLE X

PFC$_3$ STORED

Serial Passage	*Material and Dosage*	*Number inoculated*	*Results of Inoculation*	*Survival rate on Challenge*
PFC$_3$	AAF 10-^{3}	4 Fowls	D4,D4,D9,D10*	—
PFC$_3$ P$_4$	AAF 10-^{3}	4 Fowls	D4,D4,D4,D4	—
PFC$_3$ P$_{17}$	AAF 10-^{3}	4 Fowls	D4,D4,D5,D6	—
PFC$_3$ P$_{21}$	AAF 10-^{3}	6 Fowls	D6,D7,D8,D8	2/2
PFC$_3$ P$_{29}$	AAF 10-^{3}	8 Fowls	D5,D7,D8,D9	3/4
PFC$_3$ P$_{46}$	AAF 10-^{3}	8 Fowls	D6,D6,D10	5/5
PFC$_3$ P$_{61}$	AAF 10-^{3}	6 Fowls	D6, D7, D8	3/3
PFC$_3$ P$_{69}$	AAF 10-^{3}	8 Chicks (2 months)	D5,D5,D6,D6, D6,D6,D6,D9	—
PFC$_3$ P$_{86}$	AAF 10-^{3}	8 Chicks (4½ months)	D3,D6,D7,D7,D7. 320(3)	3/3

*The 2 birds of this group that died on the 9th and 10th days had been inoculated previously with PFC$_2$ P$_{11}$ C$_7$.

MOUSE-PASSAGED STRAIN OF PFC_2 P_{11} C_{30} P_{44}

Material from pigeon embryos of the passage PFC_2 P_{11} C_{30} P_{44} was inoculated intracerebrally into 12 mice on the 22nd January, 1950. During the 5 days following inoculation these mice showed no symptoms. Six of them were destroyed on the 5th day, and 10 fresh mice, and 4 chick embryos were inoculated with a suspension of the pooled brain tissues. The chick emryos died in the usual time for this strain of virus, and the fluids showed haemagglutination to normal titres. Since the 10 mice of the second direct passage showed no symptoms of illness, 6 of them were killed on the 5th day, and 3 chick embryos and a further 10 mice were inoculated with the pooled suspension of their brains. The chick embryos did not die, and the mice of the 3rd direct passage showed no symptoms of illness.

Accordingly, 10 mice were inoculated with allantoic fluid from the chick embryos that had been infected by inoculation of brains from the 1st passage mice. On the 6th day these mice of the first alternate passage (M_1 E_1 M_1) were killed and further inoculations were made in chick embryos and mice with a pooled suspension of their brains. Since the mice of this group also failed to show symptoms, a further batch of mice was inoculated on the 13th of February with the allantoic and amniotic fluids of the chick embryo, 2nd alternate passage. These mice constituted Passage M_{3A} E_{2A}. On the 5th day after inoculation two of the mice of the M_{3A} E_{2A} group were paralyzed, and one was dead. Two further mice showed paralysis on the following day. Chick embryos, inoculated with brain suspension from paralyzed mice, died within the normal period and their fluids showed haemagglutinations for fowl cells. All the mice of this group showed symptoms by the 8th day. A suspension of the brains of the mice showing paralysis on the 5th day was inoculated directly into a further group of mice, making M_1 M_{3A} E_{2A}. On the 5th day 2 mice in this group were dead and 2 showed paralysis. Brains were collected from paralyzed mice and inoculated directly into a further group of mice M_5 E_{2A}, and into chick embryos to establish the presence of virus ; 5 days later the mice of the M_5 E_{2A} passage were showing paralysis, and from this point the series was carried on directly in mice by brain-to-brain passages, with a check at each transfer by chick-embryo inoculation.

On the 1st of May, 3 pooled brains from passage M_{16} were titrated in chick embryos, and 50 susceptible birds were inoculated with 1cc of a dilution of 10^{-3} of the suspension. The dilutions in the chick-embryo titration ranged from 10^{-3} to 10^{-9} but only a few deaths occurred irregularly, and there was no evidence of haemagglutination in the fluids of any of the dead embryos. The inoculated birds remained susceptible and none showed any definite inhibition of haemagglutination.

In view of the disappointing result obtained by direct titration of the brains a further titration was carried out with allantoic fluids

of M_{17} E_3, *i.e.* with the fluids from the ordinary check-test embryos of the 17th mouse-brain passages. The dilutions in this titration ranged from 10^{-1} to 10^{-4} and the results were as follows :—10^{-1}, 3/3; 10^{-2}, 5/5; 10^{-3}, 4/5; 10^{-4}, 2/5; the fractions representing deaths over inoculated.

Titration M_{19}; Brains (Desiccated)

At the 19th passage *i.e.*, M_{19}; E_{2A} a pool of mouse brains was desiccated. The desiccated material was titrated on the 20th May, 1950, in chick embryos and fowls. In the chick-embryo titration 5 out of 5 embryos died at the lowest dilution, 10^{-3}, and there were no deaths at all in the higher dilutions. In the fowl titration 6 groups, each of 3 birds, were inoculated within the range 10^{-2} to 10^{-7}, but 15 days later none of the birds showed any inhibition of haemagglutination. They were not challenged.

The chick-embryo titration was repeated on the 31st May, and titration in mice was also attempted. In the chick-embryo titration the dilutions were begun this time at a lower level. At 10^{-2} the suspension killed 5 out of 5 embryos, at 10^{-3} 4 out of 4, at 10^{-4} 1 out of 5, and 10^{-5} 2 out of 5. In the mouse titration 5 groups, each of 10 mice were inoculated intracerebrally with a dose of 0·03cc of tenfold dilutions ranging from 10^{-2} to 10^{-6}. In the 10^{-2} group 3 mice died, one on the 4th day, one on the 6th, and one on the 10th. In the 10^{-3} group 1 mouse died on the 10th day. The remaning 46 mice survived. It seems that at this passage level there was no very great difference between mouse titres and chick-embryo titres.

Repassage M_{19} in Chick Embryos

Titration first repassage. From the titration embryos of the 10^{-3} group of the 20th May a further titration was made in chick embryos and fowls, on the 24th May, using pooled allantoic and amniotic fluids. The results of the titration in chick embryos were as follows:—10^{-3}, 5/5; 10^{-4}, 5/5; 10^{-5}, 5/5; 10^{-6}, 5/5; 10^{-7}, 5/5; 10^{-8}, 1/5. Five groups, each of 3 of fowls, were also inoculated with tenfold dilutions of the same fluids, ranging from 10^{-2} to 10^{-6}. Tested for serum inhibition on the 14th day, only 1 bird in the 10^{-2} group showed complete inhibition up to 1:160. All the remaining birds failed to inhibit in a dilution of 1:20. They were not challenged.

Titration 2nd repassage. On the 10th June, 1950, further titrations were made with pooled allantoic and amniotic fluids of the titration embryos of the 10^{-3} and 10^{-4} dilutions of the 24th May. These titration embryos represented the 2nd chick-embryo repassage of M_{19} desiccated brain tissue. Inoculated into 3 groups of 10 mice each at dilutions of 10^{-1}, 10^{-2}, and 10^{-3}, in a dosage of 0·03cc, the 2nd repassage fluids gave the following results :—

10^{-1}	D4,D5,D5,D5,D5,D5,D5,D5,D6,D6.	10/10
10^{-2}	D4,D5,D5,D5,D5,D5,D6,D6,D6.	9/10
10^{-3}	D5,D5,D5,D6,D6,D6,D6,D7.	8/10

Five groups, each of 3 fowls, were inoculated with the same material, ranging from "undiluted" to 10^{-4}. Tested on the 10th day the sera of these birds gave the following results:—

Undiluted	320 (2)	80 (1)	
10^{-1}	320 (1)	20 (1)	20 (1)
10^{-2}	320 (1)	20 (1)	20 (1)
10^{-3}	160 (1)		20 (2)
10^{-4}			20 (3)

It seems that after 2 repassages in chick-embryos the titre for mice has been raised although not nearly to the same extent as the chick-embryo titre, while there has been a significant increase in the titre for fowls.

Titration 3rd repassage. On 20th June 10 chick embryos were inoculated with the stored allantoic and amniotic fluids of M_{19}; E_3, 10^{-4}, titration embryos of the 2nd repassage 26th May, 1950. Two embryos died within 24 hours with positive fluids, and a further seven at 42 hours, also with positive fluids. A pool of these 7 embryos, of this 3rd repassage was prepared, and a series of tenfold dilutions ranging from undiluted to 10^{-4} was set up. With each of these 5 suspensions a group of 7 birds was inoculated with a 1cc dose. The response of the inoculated birds was judged by the haemagglutination inhibition test 14 days after the inoculation. The results were as follows :—

1cc Undiluted	320 (5)	160 (1)	20 (1)	
1cc x 10^{-1}	320 (1)	80 (1)	20 (5)	
1cc x 10^{-2}	320 (2)	160 (2)	80 (1)	20 (2)
1cc x 10^{-3}	320 (1)	40 (1)	20 (5)	
1cc x 10^{-4}			20 (7)	

The birds were not challenged but if one can judge from the inhibition titres it seems that the attacking power for fowls had been slightly raised from its original level by the three repassages in chick-embryos.

Titration of M56 E3. The direct mouse-passaged series was carried to the 56th mouse-passage, and on 25th Jan., 1951, material from chick-embryos inoculated with brain suspension of M56E3 was titrated in tenfold steps from 10^{-1} to 10^{-6} with 5 embryos in each group. All the inoculated embryos died with positive fluids, but deaths were delayed in the 10^{-6} group, indicating that the end point was being approached. Simultaneously a group of 8 susceptible fowls was inoculated with 1 c.c. of 10^{-2} dilution of the same pool. Only 1 bird out of the 8 inhibited haemagglutination when the sera were tested 14 days after inoculation.

Repassage of M56E4 in pigeon-embryos. Material from titration embryos of M56E3 was transferred to pigeon-embryos on 4/3/51. The strain appeared to be unchanged in virulence for pigeon-embryos during 9 pigeon-embryo repassages. At the 6th repassage

a titration of embryonic fluids in chick-embryos gave $LD_{50} = 10^{-8}$ but of 8 fowls inoculated with $1ccx10^{-2}$, only one developed a significant level of inhibition.

Similarly after 6 further repassages in chick-embryos the chick-embryo titre of fluids was $LD_{50} = 1c.c. \times 10^{-8 \cdot 3}$, but of 8 fowls inoculated with $1c.c. \times 10^{-3}$,3 developed inhibitory titres of 40 and the remaining 5 showed no rise.

At the 7th pigeon-embryo repassage there was a sudden return of virulence, and $1cc \times 10^{-3}$ of pooled allantoic fluids killed 6 birds out of 11 inoculated, immunizing the survivors. A test made with pooled embryo on the fluids of the 9th pigeon-embryo passage gave 3 deaths out of 11 birds inoculated, an indication that virulence might possibly again fall with successive pigeon-embryo passages, but the strain could not be carried on in the pigeon-embryo.

THE "D" STRAIN OF VIRUS

In December, 1950, a strain of fowl-plague virus was isolated from the brain of a bird apparently in the final comatose state of the disease. This bird came from a small flock of about 30 European cross-bred fowls kept on the roof on an apartment house in the city of Cairo. The Veterinary Inspector who had been called in by the owner reported that 8 deaths had occurred in the flock within a few days and that all the remaining birds were showing symptoms of illness at the time of his first visit. The most severely affected living bird showed all the classical symptoms of an acute case of fowl plague. A 10 per cent suspension of brain tissue in normal saline was prepared in the usual manner and four 10-day-old chick embryos were inoculated into the allantoic cavity. All four embryos were dead by the 42nd hour, and pooled embryonic fluids were used to identify the virus, by haemagglutination and the inhibition test with fowl-plague and Newcastle-disease immune sera. The pooled allantoic fluids of these embryos and the original brain suspension were stored at –20°C, for later use.

After 64 days storage the allantoic fluids were passaged once in four 10-day-old chick embryos. All embryos were dead by the 42nd hour, and after a rough titration of virus in the pooled fluids by haemagglutination, 17 birds were inoculated intramuscularly with $1cc \times 10^{-5}$ of these fluids. As judged by serum-inhibition test prior to the challenge inoculation 5 of the 17 birds were immune as a result of treatment with experimental vaccines, and 12 were fully susceptible. It was the intention to use the 12 susceptible birds to initiate an outbreak of fowl plague in an experimental flock of 50 susceptible birds, and they were placed in the pen with the 50 susceptibles. None of the birds showed any symptons of illness after the inoculation, and the inhibition titres of the sera of the susceptible birds rose to the following levels, 320 (7 birds), 160 (3 birds) 80

(1 bird) and 20 (1 bird). None of the 50 contact birds showed any rise in titre.

Four days after the inoculation, when it was seen that none of the inoculated susceptible birds were showing signs of illness, 3 further susceptibles were inoculated with a larger dose, (1 cc. x 10^{-2}) of the same pooled embryonic fluids. All three survived without any apparent signs of illness. Two developed inhibiting titres of 320, and one a titre of 160. When the second group also failed to show symptoms within 6 days a further passage was made on 11/3/51 of these embryonic fluids. The embryos again died within 42 hours, and 3 susceptible fowls were inoculated with 1 cc. x 10^{-2} of pooled embryonic fluids. This represented the 3rd passage (DF3). Again the inoculated birds remained healthy and inhibition titres rose to 320 (2 birds), 80 (1 bird).

Two days later the original brain emulsion was removed from the deep-freeze cabinet, after 88 days storage, and inoculated into chick-embryos and into 3 susceptible birds in doses of 0·5 cc. x 10^{-1}. The three birds survived but there was no rise in the inhibiting titre of their sera, which remained at <20. Later they were proved to be susceptible. The embryos were dead by the 42nd hour, and the pooled allantoic fluids were shown by haemagglutination test to contain virus to about normal titre. Three susceptible birds, inoculated with 1 cc. x 10^{-3} of pooled allantoic fluids from these embryos, died on the 3rd, 4th and 6th days after inoculation, with symptoms and lesions of acute fowl plague, and virus was recovered from their brains.

We had now at our disposal two separate lines of the D virus, one of which after a single passage in chick-embryos and subsequent storage for 64 days at −20°C, had proved in the next two chick-embryo passages to be apathogenic for chickens, but highly immunogenic, immunizing 17/18 susceptible birds without any symptoms of illness, and the other, derived from the original brain suspension stored for 88 days, which after a single chick-embryo passage was apparently of normal virulence for fowls. It was decided to passage each of these lines in chick and pigeon-embryos, beginning in the one case with the 3rd and in the other with the 1st chick-embryo passage.

After 3 passages in pigeon embryos the line derived from the stored embyonic fluids, which for convenience has been termed DF, killed two out of four birds on the 10th day, immunizing the survivors. The same line passaged in chick-embryos only (DF6) immunized all four birds inoculated, although two of them were dull or slightly ill for 3 days. After 6 passages in pigeon embryos this line killed 9 out of 9 birds and the corresponding passage in chick-embryos 8 out of 9 birds inoculated. After 25 pigeon-embryo passages 4 birds out of 10 survived and were immunized, while

with the corresponding chick-embryo passage (DF28) three birds survived out of 10. With the other line derived from the stored brain suspension the 6th pigeon-embryo passage killed one bird out of 4 and the corresponding chick-embryo passage (DF6) two out of four.

At this stage we were compelled to abandon the passaging of one line owing to shortage of pigeon embryos during the summer months, and we elected to continue the DF line only, which line was carried to the 25th pigeon-embryo passage and the 28th chick-embryo passage with the results that have already been indicated, before the senior author left Cairo. For convenience of reference the records of the DF and DB lines have been tabulated (Table XI).

TABLE XI

Serial passage	*Number inoculated*	*Dose* 1cc	*Results*	*Serum-inhibition titres*
			DF LINE OF 'D' VIRUS	
DF_2	12	10^{-5}	No symptoms	320(7) 160(3) 80(1) <20 (1)
DF_2	3	10^{-2}	No symptoms	320(2)160(1)
DF_3	3	10^{-2}	No symptoms	320(2) 80(1)
DF_3P_3	4	10^{-2}	D10 D10	80(2)
DF_6	4	10^{-2}	2 birds sick 6th 9th days.	320(2)160(1) <160(1)
DF_3P_6	9	10^{-2}	$D_1D_2D_2D_4D_4D_5D_5D_6D_7$	
DF_9	9	10^{-2}	$D_1D_1D_2D_4D_5D_7D_7D_8$	320(1)
DF_3P_{25}	10	10^{-2}	$D_4D_5D_5D_6D_8D_8$	320(4)
DF_{28}	10	10^{-2}	$D_4D_5D_5D_6D_7D_8D_{10}$	320(3)
			DB LINE OF D VIRUS	
DB_1	3	10^{-3}	$D_3D_4D_5$	
DB_1P_6	4	10^{-2}	D_8	320(3)
DB_6	4	10^{-2}	D_6D_8	320(2)

INTERFERENCE BETWEEN NC AND FP VACCINES

Preliminary observations, both in chick embryos and adult birds, gave clear indications of the existence of mutual interference between vaccine strains of Newcastle-disease and fowl-plague viruses. Since, in the event of a satisfactory avirulent vaccine strain being produced, it might be desired to immunize birds simultaneously against both diseases, two small trials were made in the

laboratory of the effect of inoculating birds simultaneously with the two living vaccines.

In the first trial 7 birds, susceptible to both viruses, were inoculated with a mixture containing the equivalent of the usual dose of each of the vaccines. The Newcastle disease vaccine was $Nc_3\ PR_4$, a dried preparation of the Komarov strain, titre in chick embryos $LD50 = 10^{-8 \cdot 4}$, which immunized susceptible birds regularly in doses up to 1cc x 10^{-5} and an occasional bird up to 1cc x 10^{-8}. The fowl-plague virus was desiccated (B) $PFC_{34}\ P_{15}\ C_6\ P_{25}$ chick-embryo titre, $LD50 = 1cc \times 10^{-7 \cdot 9}$, which immunized birds regularly at 1 cc x 10^{-3}, but fell off rapidly in performance in higher dilutions (Table 12). It will be observed that in two birds only out of the 7

Table XII

Fowl Number	*Newcastle-disease CCA Inhibition*		*Fowl-plague CCA Inhibition*		*Challenge*	
	Initial titre	*Post vaccination titre*	*Initial titre*	*Post vaccination titre*	*Challenge fowl-plague virus*	*Challenge Newcastle-disease virus*
560	0	160	0	20	D 4	—
564	0	320	0	20	D 5	—
565	0	320	0	20	D 6	—
568	0	320	0	0	D 4	—
569	0	320	0	20	D 6	—
240	0	160	0	320	Immune	Immune
243	0	0	0	320	Immune	D 4

that received both viruses was protection given against challenge with fowl-plague virus. One of these two (No. 240) was immunized against both viruses, but the other bird (No. 243) remained susceptible to Newcastle disease. The five birds that remained susceptible to fowl plague appear to have been immunized against Newcastle-disease, judging by the sharp rise in the inhibition titres of their sera. Twenty-three birds immune to Newcastle disease were inoculated at the same time, 17 with fowl-plague vaccine alone and 6 with the same mixture of Newcastle and fowl-plague vaccines. One died on the 4th day after vaccination, and one other bird (No. 572) with a post-vaccination titre of 1:80 against fowl-plague virus, was ill from the 6th to 10th days after challenge with fowl-plague virus. The remaining 21 birds all became immune to fowl plague and resisted challenge with a virulent strain.

A second trial was made with two other batches of vaccine, Another strain of fowl-plague vaccine (PCF^{30}; P^{85}) was employed in combination with Batch Nc P_3 R_6 of Newcastle vaccine. Batch PFC_{30} P_{85}, desiccated, LD50 titre for chick embryos 10^{-8}, immunized birds regularly at 10^{-3} and occasional birds up to 10^{-5}. Nc P_3 R_6, desiccated, LD50 chick embryos $10^{-8.75}$, immunized two out of three birds at 10^{-5}, and none out of 3 at 10^{-7} and 10^{-9}.

This particular batch of Newcastle vaccine (Nc P_3 R_6) was selected for the trial because it appeared to be weaker than the average in immunizing power, although its titre for chick embryos was normal. However, since the respective chick-embryo titres indicated roughly a sixfold advantage of numbers in favour of the Newcastle vaccine, an attempt was made in two out of the three groups to give an advantage to the fowl-plague strain, in one group by increasing the dose of the fowl-plague vaccine tenfold, and in the other by reducing the dose of Newcastle vaccine tenfold. The results are tabulated (Table XIII).

Table XIII

			NEWCASTLE-DISEASE		*FOWL PLAGUE*	
	Dosage 1 cc x	*Fowl Number*	*Initial titre*	*Post vaccination titre*	*Initial titre*	*Post vaccination titre*
		627	0	320	0	320
Nc P_3 R_6	10^{-3}	629	0	160	0	320
PFC_{30}; P_{85}	10^{-3}	631	0	320	0	40*
		632	0	320	0	20*
		621	0	320	0	320
Nc P_3 R_6	10^{-4}	622	0	320	0	320
PFC_{30}; P_{85}	10^{-3}	623	0	320	0	320
		625	0	320	0	320
		626	0	320	0	320
		633	0	320	0	320
Nc P_3 R_6	10^{-3}	634	0	20	0	320
PFC_{30}; P_{85}	10^{-3}	635	0	80	0	320
		636	0	320	0	0*
		638	0	320	0	320

The three birds marked with an asterisk, 631, 632 and 636, were subjected to a challenge inoculation with fowl-plague virus (1cc x 10^{-3} allantoic and amniotic fluids) on the 20th day after vaccination.

Three susceptible control birds died on 4th, 7th and 9th days after the challenge, but birds 631, 632 and 636 survived. Bird 636 was definitely ill on the 4th, 5th and 6th days after challenge, and by the 12th day its serum reached a haemagglutination inhibiting titre of 320. 631 and 632 showed no symptoms after challenge but the serum titre of 631 rose to 320 on the 12th day and that of 632 to 80. Apparently the challenge virus was able to establish itself and to multiply in all 3 birds, and accordingly all of them must be regarded as only partially immune to fowl plague.

It is clear from the result of this small trial that simultaneous immunization against the two diseases is practicable, and that, all that is necessary is to bring the dosages, in terms of attacking power for birds, very roughly into balance.

DISCUSSION

Despite the fact that for some years past an epizootic of Newcastle disease has each year raged over most of the irrigated area of Egypt, outbreaks of fowl plague have continued to occur; and in a brief survey of the incidence of the two diseases in table birds brought into Cairo from Upper Egypt in the spring of 1951, roughly 15 per cent of the viruses isolated proved to be fowl plague (Bayoumi and Escarous, 1952). Since 1947 routine immunization against fowl plague in Egypt has been by single subcutaneous injection of 0·5 cc. of a lanolin and oil emulsion of formalized suspension of virus in the form of whole egg content with *Mycobacterium butyricum* as adjuvant. The quanitity of whole egg content in each dose of vaccine is only 0·1cc. so that an average Egyptian egg will yield from 250 to 280 doses of vaccine. Nevertheless, one cannot fail by contrast to be impressed by the relative ease with which millions of doses of desiccated living virus vaccine against Newcastle disease can be prepared, where the yield from a single egg is roughly 100 times greater, as well as by the facility with which outbreaks of the disease are extinguished by the living virus vaccine. Our interest in variants or mutants of the fowl-plague virus derives primarily from these considerations.

The literature on variation of the virus of fowl plague has been reviewed by Moses, Brandly, Jones and Jungherr (1948) and Daubney, et. al (1949). Most of the studies on variation have been directed to the isolation of variants or mutants that might be utilized as living vaccines; but neither the mouse-passaged strains of Schmidt and Oersko (1935) and MacKenzie and Findlay (1937) nor the tissue-cultured strain of Hallauer (1939), showed much promise in that direction.

Jungherr, Tyzzer, Brandly and Moses (1946) reported the isolation of two variants of the Dutch East Indies strain of virus from embryos inoculated with infectivity-end-point dilutions. In 1948, Moses, Brandly, Jones and Jungherr stated " The relative avirulence and immunogenicity of the variants for chickens pointed to their possible value as immunizing agents for use in the living state, either alone or following the injection of killed virus vaccines ". These variant strains produced lethal infection in chick-embryos, but the survival time of the embryos was about twice that of embryos, inoculated with the parent strain of virus. In 16-day embryos they state " multiplication of the variant virus was either completely suppressed or its viability destroyed. Amounts of virus that were 100 to 1,000 times greater than those required to kill the younger embryos were found, however, to infect lethally the 16 day old eggs following a.c. injection." Concentration of variant virus in the embryonic fluids was 100 to 1,000 times less than that of the parent strain. Once isolated these variants appeared to be stable in their characters through 10 to 15 passages in embryos that were injected with 10 to 100 m.i.ds. In one group of trials the authors succeeded in immunizing all birds treated with amounts of variant virus ranging from 2 to 2 x 10^6 embryo m.i.d's., the variant virus causing a mortality of 4 out of 27 birds inoculated. In trials with the other variant in doses ranging from 10 m.i.d. (chick-embryo) to 10^6 m.i.ds. 16 out of 21 birds were immunized without mortality. In summing up they say " The living variant viruses were capable of provoking a high grade immunity when injected into susceptible chickens, and when introduced onto the surfaces of the nasal passages, cloaca and feather follicles. The dosage within a wide range was of little or no consequence on the resultant immunity. Apparent differences in the immunizing properties were demonstrated between two variant strains." It might be remarked that in the table illustrating immunization by these various routes only 2 control birds out of 8 showed symptoms following a challenge inoculation. Possibly the remainder had been immunized by contact with the vaccinated birds.

Although the studies reported in the present paper are incomplete it was thought desirable that they should be placed on record when the senior author left Egypt. They show that attempts to isolate "variants" or mutants from recently collected field strains of virus do at times meet with success. Serial cultivation in the pigeon-embryo seems to afford an environment suited to the selection of certain mutants of reduced pathogenicity for fowls and indeed may actually induce such mutations. Of the four field strains that are considered in this paper, two failed to yield any recognizable mutant during serial passage in pigeon and duck embryos. Another, after as little as eleven serial passages in pigeon embryos, yielded a mutant that was immunogenic and apathogenic for fowls, but

within the next 2 or 3 passages in either pigeon or chick embryos the strain entirely lost its capacity to infect or multiply in fowls, and this capacity was only regained after 27 to 30 serial repassages in the chick-embryo. Throughout this period the strain continued to kill chick-embryos in 42 hours, and to reach concentrations of the order of 10^8 m.i.ds. for chick embryos in the embryonic fluids. Once the capacity to infect fowls had been reacquired it was retained unimpaired by three separate lines during 95 to 120 further serial passages in pigeon embryos.

Another series, started by one preliminary passage in chick embryos of the stored material of the 2nd chick-embryo passage, from which the first series had been derived, gave quite different results in that capacity to infect chickens was never lost, and after 84 serial passages in pigeon-embryos the strain was still lethal to 5 out of 8 inoculated birds.

A third line, started with fully virulent stored material of the 6th pigeon-embryo passage of the original series, maintained its capacity to infect birds through 125 pigeon-embryo passages, during which its pathogenicity for chickens fell gradually to the point where it killed about 10 per cent of inoculated fowls.

Of the three lines eventually derived from the original avirulent 11th pigeon-embryo passage, the one recovered from the 30th chick embryo repassage, still killed 1 out of 20 birds at the 125th pigeon-embryo passage. Of the other two, derived from the 34th chick-embryo repassage, one showed a considerable exaltation of virulence between the 40th and 80th pigeon-embryo repassages, which caused us to suspend the series. The other line, which after 15 repassages in the pigeon-embryo was unintentionally passed six times in chick embryos as a mixture with a virulent strain of Newcastle disease virus and then returned in pure culture to pigeon-embryos, behaved quite differently, (see Table VII and VIII). By the 40th pigeon-embryo passage the one line had reacquired high virulence for chickens, whereas the other at the equivalent level in pigeon-embryo passages (BP15C6P25) immunized 15/16 birds without mortality, and it remains after 95 pigeon-embryo passages the most useful and least pathogenic of the variants. Antigenically the 'B' strain is unchanged, as judged by haemagglutination-inhibition and challenge of immunized birds with stock virulent virus; but the possibility that useful results might again be obtained by passaging a mixture of the two viruses is one that should not be neglected.

The few observations that we are able to record with the fourth field strain (D) emphasize how unpredictable the behaviour of a freshly isolated strain may be. Storage of the original virus in the form of brain material apparently caused a considerable fall in

virus concentration, since it is the usual experience that a 10 per cent suspension of brain tissue from an acute field case is infective in high dilution. The surviving virus particles, however, seemed to be of normal field virulence judging by the result of inoculating birds with 1st passage embryonic fluids. By contrast, when 1st passage embryonic fluids were stored for 66 days, 2nd and 3rd passage fluids immunized birds without causing any symptoms of illness. Further serial passages in either chick-embryos or pigeon-embryos progressively raised the virulence of the avirulent line until by the 9th passage the mortality was 90 per cent to 100 per cent. By the 25th passage in both chick and pigeon-embryos the virulence showed a tendency to fall again, with 4 survivors out of 10 in the one case, and 3 out of 10 in the other. The virulent line derived from the stored brain suspension on the other hand rapidly lost virulence under the same treatment, killing only half the birds after 6 passages in chick or pigeon-embryos, at which point the work ceased. Prolonged cold storage has been associated with type mutation of foot-and-mouth disease virus (Trautwein, 1929; Daubney 1934). The senior author has also observed that prolonged storage at -40°C of strains of rinderpest virus attenuated by yolk-sac cultivation leads to a slight but clearly recognizable exaltation in the virulence of the strains for cattle (unpublished data). Such happenings are usually attributed to a differential in survival rate of particles of different virulence or antigenic make-up; but it is interesting to see this factor apparently exercising a selective effect in one only out of two suspensions of the same fowl plague virus, separated only by one passage in chick-embryos and a difference in the substrate carrying the virus during storage.

The rapid exaltation of virulence for the normal host of the apathogenic strain recovered from the stored allantoic fluids, observed during serial passage in a host (pigeon-embryo) that on the basis of previous experience might be expected to favour "attenuation," is a phenomenon that is by no means uncommon. There are indications of a similar change in the records of PFC2 P11C30 and PFC2P11C34 during early repassages in pigeon-embryos (Tables VI and VII), and a further clearcut example in the records of the mouse strain derived from C30P44, when after 56 intracerebral passages in mice it was submitted to serial repassage in pigeon-embryos. A similar exaltation of virulence for the normal host has been recorded by Daubney and Hudson during serial goat-passage of field strains of rinderpest virus, initially of abnormally low virulence for cattle. The recovery of virulence may at times be so sudden and unexpected as to evoke doubts as to the reliability of the results; and it is possible that for this reason such incidents may not be recorded.

Apart from the sudden access of virulence in the pigeon embryo

repassaged line of the 56th mouse passage, there is little that calls for comment in the mouse-passaged line. It was only after several passages had been made that moderate titres were reached in the brains of the mice, and even then titres for mice continued to be fairly low. Repassage in chick embryos raised chick-embryo titres, and apparently mouse titres also, but infective titres and immunogenic capacity for fowls remained low until the 7th pigeon-embryo repassage, when the sudden return of virulence was remarked. It might be pointed out that the level of virulence reached in this sudden mutation was probably far higher than that of the pigeon-embryo passage from which the mouse strain was originally derived (see Table VI). Possibly from this point onwards the strain might have fallen rapidly in virulence during further repassage in pigeon embryos, but at the time it was not possible to continue the series.

Fowl-plague virus is usually considered to be one of the more stable viruses. Its host range is a restricted one, and strains varying in antigenic make-up from the original type-strains have never been recognized. But from the experiences recorded in this paper it seems highly probable that simple laboratory manipulation of freshly isolated field strains of virus will lead to the isolation of other variants or mutants, some of which may prove to be of even greater value than the B strain for the immunization of fowls.

SUMMARY

Two out of four recently isolated field strains of fowl-plague virus failed to yield recognizable mutants during serial cultivation in pigeon and chick embryos.

One strain became avirulent for fowls at the 11th pigeon-embryo passage, but capacity to infect fowls was completely or almost completely, lost during the next three passages in either chick or pigeon embryos, and was not regained until 27 to 30 additional passages had been made in chick embryos. The regained capacity of this strain to infect fowls was unaffected by further serial repassage in pigeon embryos.

Other lines of this strain, started from material stored before any mutation had occurred, behaved differently under serial cultivation in pigeon embryos. One line remained lethal to rather more than 50 per cent of inoculated birds, while the virulence of a second line fell to a level at which it caused a mortality of about 10 per cent.

Of two lines, derived from the series in which infectivity for fowls had been lost and reacquired, one showed an exaltation of virulence after further serial pigeon-embryo passage. A substrain "B" of this line, which had been passaged six times in chick

embryos simultaneously with Newcastle-disease virus, was of remarkably low virulence and high antigenic capacity for fowls from that point onwards. The second line, subjected to 125 serial passages in the pigeon embryo was at that stage lethal to about 5 per cent of inoculated fowls.

A substrain derived from one of the pigeon-embryo passaged strains by serial intracerebral passage in mice showed greatly reduced capacity to infect and immunize fowls. After 7 repassages in the pigeon embryo there was a sudden access of virulence for fowls.

When the first passage in chick embryos of a fourth field strain of virus was stored at –20°C for 64 days, and then passaged in chick embryos, material from two successive passages immunized birds without causing any illness; but its full virulence was restored after a further 6 passages in chick or pigeon embryos. After a further 19 passages the virulence showed signs of declining. Virus stored in the original brain suspension for 88 days was still fully virulent after a single passage in chick-embryos, but showed signs of declining after 6 further passages in chick or pigeon-embryos.

Mutual interfence was demonstrable in birds inoculated with mixtures of " attenuated " fowl-plague and Newcastle-disease viruses, but when titres of the two viruses were adjusted so that neither virus had a marked advantage in numbers most of the birds developed immunity to both diseases.

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A SEMI-MICRO METHOD FOR THE ESTIMATION OF SERUM CALCIUM BY DIRECT TITRATION

By

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Introduction

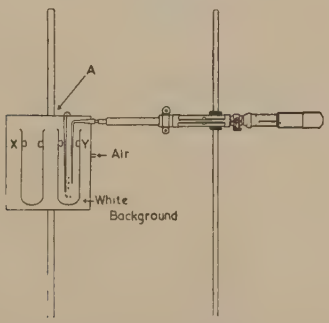
The estimation of calcium in blood serum by direct titration with disodium ethylenediamine tetra-acetate has been described by Greenblatt and Hartman (1951), Holtz and Seekles (1952) and Elliot (1952). The titration is carried out at a pH about 12, using a solution of the dye 'murexide' as indicator. The method is much more rapid than the standard oxalate precipitation techniques, and where comparative studies on the two methods have been made, the agreement of analytical results has been found to be satisfactory.

The chief difficulty in the direct titration method is that of obtaining a satisfactory end-point, as the colour change of the indicator from red to blue-violet is not particularly sharp. Holtz and Seekles (1952) suggest the use of a photoelectric colorimeter for checking the colour change at the end-point. The difficulty of obtaining a good visual end-point is seriously aggravated in the case of sera which are haemolysed or highly pigmented.

The method described here is a semi-micro one which enables a determination to be carried out on 0·50 ml. of serum and minimizes the difficulty involved in obtaining a satisfactory visual end-point.

Methods and Materials

Fig. 1



Micro-titration Apparatus

Apparatus. The apparatus used is shown in Fig. 1. 'A' is a titration stand with clips mounted on a white background at 'X' and 'Y' for holding the titration tubes. The micro-burette, an 'Agla' micrometer syringe (Burroughs Wellcome and Co.), is mounted as shown on a retort stand and can be refilled in situ.

Reagents. A 0·004 M solution of disodium ethylenediamine tetra-acetate (L. Light and Co.) was used. This solution was checked against a calcium standard (10 mg.Ca/100 ml.), prepared by dissolving 250 mg. pure $CaCO_3$ in the minimal amount of 0·1 N-HCl and making up to 1 litre.

The indicator was prepared by shaking up excess 'murexide' with distilled water and filtering, a fresh solution being prepared each week.

Experimental procedure. The following are carefully pipetted into a titration tube ($3\frac{1}{2}$ in $\times\frac{3}{4}$ in. test tube): 0.5 ml. serum (or standard calcium solution), 10 ml. distilled water, 0.2 ml. 4N-NaOH, 0·10 ml. 'murexide' solution, 1 drop capryl alcohol.

The tube is then placed in the titration stand at position 'Y' and the level of the micrometer syringe adjusted until the end of the titration tip is about half an inch below the surface of the solution. During the titration stirring is effected by bubbling a stream of air through the solution, the capryl alcohol preventing any foaming.

It is advisable first to titrate a sample of the calcium standard which is then transferred to position 'X' and used as a control in the titration of the samples of serum. In this way one can ensure that calcium standard and serum samples are titrated to the same end-point. The titration should be carried out in good daylight.

As mentioned earlier, it is almost impossible to carry out the direct titration on haemolysed samples of serum. In such cases the authors have found it convenient to precipitate the proteins from such samples and carry out the estimation on the colourless protein-free solution. The procedure is as follows: Pipette 0·625 ml. of serum into a 15 ml. conical centrifuge tube, make up to 5.0 ml. with distilled water and add 5·0 ml. of 10 per cent trichloroacetic acid. Mix by inversion, centrifuge and pipette 8·0 ml. of the protein-free supernatant ($\equiv$0·5 ml. serum) into a titration tube. Add 1·0 ml. 4N-NaOH, 2·0 ml. distilled water, 0·10 ml. 'murexide' solution and titrate as before. The extra NaOH added in this case is to allow for the neutralisation of the trichloroacetic acid. A sample of the calcium standard is treated in exactly the same way.

RESULTS

In order to compare the results obtained by the above method with those given by the Clark and Collip (1925) procedure, a number of sera were analyzed for calcium by both methods. The results are shown in Table 1, and the agreement between the two methods is in most cases satisfactory. Both methods were used also in the analysis of sera from thirty normal horses. The direct titration method gave a mean of 12·4 mg./100 ml. with a S.D. of 0·96, while the Clark and Collip method gave a mean of 11·8 mg./100 ml. with a S.D. of 1·16.

The slightly higher figure given by the direct titration method is probably due to the yellow pigmentation of the horse sera which leads to a tendency to 'over-titrate.'

TABLE 1.

COMPARATIVE FIGURES FOR SERUM CALCIUM BY CLARK & COLLIP AND DIRECT TITRATION METHODS

Clark & Collip (*mg.*/100 *ml.*)	*Direct titration* (*mg.*/100 *ml.*)
9·1	8·8
9·4	9·2
10·0	9·8
11·6	12·0
12·1	12·3
12·1	11·9
12·3	12·7
11·9	12·4
12·3	12·3
11·9	12·8

TABLE 2

SERUM CALCIUM VALUES OBTAINED BY THE DIRECT TITRATION METHOD, WITH AND WITHOUT REMOVAL OF PROTEINS

Direct Titration on serum (*mg.*/100 *ml.*)	*Direct Titration on protein-free solution* (*mg.*/100 *ml.*)
5·6	5·5
3·4	4·2
10·5	9·9
9·5	9·0
5·6	5·6
9·8	10·9
10·9	11·2
11·0	11·3

Table 2 gives comparative results on a number of sera obtained with and without the removal of proteins. Although Holtz and Seekles (1952) state that the removal of proteins is not desirable, it is evident that the results obtained in this way are quite adequate for clinical purposes.

CONCLUSIONS

A semi-micro method for the direct titration of calcium in blood serum is described.

The apparatus and procedure obviate some of the difficulties associated with the end-point in the reaction.

The application of the method to haemolysed samples, after removal of proteins, is described.

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LEVELS OF SOME CHEMICAL CONSTITUENTS IN NORMAL HORSE SERA

By

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INTRODUCTION

The chemical analyses reported in this paper are the result of an investigation on the sera of thirty normal horses from a heavy draught horse stable in Glasgow. Determinations have been made of serum proteins (albumin and total globulin), alkaline phosphatase, bilirubin, calcium, magnesium, inorganic phosphate and urea.

Estimations of some of these constituents have already been carried out by a number of workers, but in the majority of cases the investigation has been confined to one or two constituents relevant to the problem being studied, and many of the data available are a by-product of other research.

Westermarck (1942, 1952) has determined calcium, inorganic phosphate and total protein in the sera of normal Finnish horses. Bonsmann and Weisner (1951), Howe (1925), and Gilman (1952) have estimated total serum proteins. Values for the relative amounts of albumin and globulin have been obtained electrophoretically (Svensson, 1946; Deutsch and Goodloe, 1945) and by sodium sulphate fractionation (Gilman, 1952). Although calcium and inorganic phosphate levels in equine sera have been extensively investigated (for references and figures see Westermarck, 1952), there appears to be no published data on the serum alkaline phosphatase of the adult horse. The normal level of serum bilirubin in the horse has been determined by Vilches (1949).

In view of the importance of measurements of the above constituents in clinical work, their possible interrelation, and the changes associated with various pathological conditions, it was considered desirable to make the present survey on a group of normal adult animals.

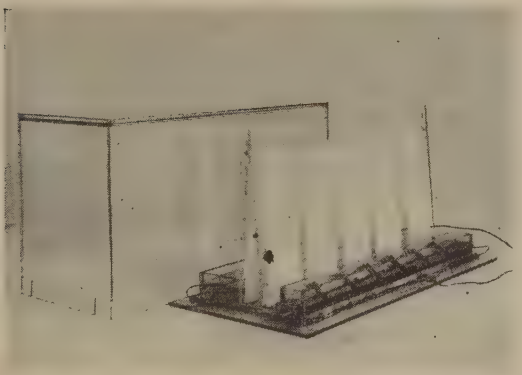
METHODS

Total protein. Total protein in the sera was determined by the modified biuret method of Wolfson, Cohn, Calvary and Ichiba (1948). The method is rapid and was found to be reliable if carried out on fresh serum and if checked frequently by Kjeldahl analysis.

Albumin and total globulin. For reasons which will be dealt with in the discussion, the relative amounts of albumin and globulin were determined by a paper electrophoresis method similar to that described by Durrum (1950), using the quantitative elution technique of Cremer and Tiselius (1950).

The apparatus used is shown in Fig. 1. It consists of a 'perspex' tank divided into five compartments, with a glass bridge to support the paper. The cover is also of 'perspex' and is removed completely when papers are

Fig. 1

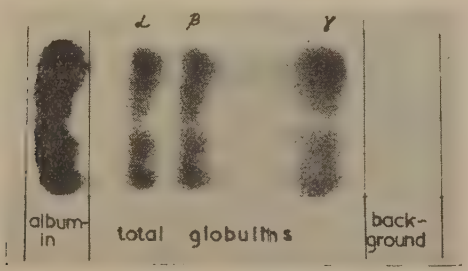


Paper electrophoresis tank with lid removed and 7 cm. strips in position.

being applied or taken off. This apparatus, though essentially similar, is much more convenient to use than that described by Flynn and De Mayo (1951).

Strips of Whatman 3 mm. filter paper 3 cm. wide (and in some cases 7 cm. wide) were used, and the serum applied by the standard Durrum technique, 0.05 ml. serum being added to the narrow strips and 0.10 ml. to the wide ones. The development with bromophenol blue reagent and the washings were carried out exactly as described by Flynn and De Mayo (1951). After drying, each strip was divided into three measured portions as shown in the photograph (Fig. 2), i.e. albumin, total globulin and a

Fig. 2.



Paper electrophoresis pattern of normal horse serum, indicating the method of sectioning

piece containing no protein for the determination of the 'background' concentration of the dye. After cutting out, the three sections were transferred to conical flasks and treated with a measured volume of a saturated solution of sodium carbonate in 50 per cent V/V methanol-water (30 ml.

for the narrow strips and 60 ml. in the case of the wide ones). After extraction for 1 hour with the alcoholic sodium carbonate, the extracts were read in an E.E.L. photoelectric colorimeter using yellow filter No. 626 (560 to 580 mμ). The 'background' reading, corrected for length, was then subtracted from the readings for albumin and globulin. The relative amounts of albumin and globulin were obtained by comparing the net readings of albumin and globulin, after multiplying the globulin figure by 1.6 (Cremer and Tiselius, 1950) to correct for its lower binding capacity for the dye. The absolute amounts of albumin and globulin were calculated from the total protein concentration and the albumin-globulin ratio.

Alkaline phosphatase. The method used was essentially that described by King (1951), except that the reaction was carried out at pH 9 using a barbitone buffer. The results are expressed in 'King-Armstrong' units, i.e. the amount of enzyme which will liberate 1 mg. phenol from disodium phenyl phosphate substrate at pH 9 in 30 minutes at 37°.

Bilirubin. Serum bilirubin was determined according to the method of Malloy and Evelyn (1937), the 'direct' bilirubin being read at 15 minutes.

Urea. Urea was determined by the liberation of ammonia by urease, and estimation with Nessler's reagent as described by King (1951).

Calcium. Serum calcium determinations were carried out by the direct titration method described by Mulligan and Jennings (1953).

Magnesium. Serum magnesium determinations were carried out using Titan Yellow by the method described by Heagy (1948).

Inorganic Phosphorus. Serum inorganic phosphorus was estimated by the phosphomolybdate method of Fiske and Subbarow (King, 1951).

RESULTS

The results of the chemical analyses on normal horse sera with mean values, standard deviation and range are shown in Table 1.

Serum Proteins

It is generally agreed that the earlier 'salting out' techniques employed for the fractionation of human serum proteins do not give a true measure of the albumin (see review by Gutman, 1948). This became evident with the development of electrophoretic methods for the fractionation of serum proteins. Recently there has been a trend towards the development of 'salting out' methods which will give results comparable to those obtained by electrophoresis. One such method, applicable to human serum, is described by Wolfson, *et al.* (1948). Because of qualitative and quantitative differences between the proteins of human serum and those of the sera of domestic animals (Edsall, 1947), a method which gives reliable results with human serum cannot necessarily be applied without modification to the sera of domestic animals. No comparison is yet available for salt and electrophoretic analysis made side by side on the same series of animal sera (see Brooksby, 1946).

Recently the technique of paper electrophoresis has been extensively used in the analysis of human serum (Cremer and Tiselius,

TABLE I

RESULTS OF CHEMICAL ANALYSIS OF NORMAL HORSE SERA

Horse No.	*Total Protein*	*Albumin*	*Total Globulin*	*A/G Ratio*	*Total Bilirubin*	*Direct Bilirubin*	*Alk. Phosphatase*	*Calcium*	*Magnesium*	*Inorg. P.*	*Urea*
	(*g/100 ml*)	(*g/100 ml*)	(*g/100 ml*)		(*mg/100 ml*)	(*mg/100 ml*)	(*King-Armstrong Units*)	(*mg/100 ml*)	(*mg/100 ml*)	(*mg/100 ml*)	(*mg/100ml*)
13	7·5	3·3	4·2	0·8	0·8	0·20	11·2	12·0	2·9	5·6	35
16	7·0	3·8	3·2	1·2	1·7	0·35	7·1	12·3	2·7	3·9	41
88	6·8	3·2	3·6	0·9	0·9	0·30	9·2	11·9	2·7	3·0	32
96	6·6	3·1	3·5	0·9	0·9	0·30	6·0	12·7	3·2	2·7	39
128	7·2	3·0	4·2	0·7	0·8	0·30	9·6	12·4	2·9	3·0	35
178	6·8	3·0	3·8	0·8	0·8	0·30	6·7	12·3	3·1	5·1	41
180	6·4	2·4	4·0	0·6	1·2	0·30	8·3	12·8	2·5	3·0	41
188	7·1	3·2	3·9	0·8	0·8	0·20	7·8	13·0	2·9	4·0	41
601	6·0	2·7	3·3	0·8	0·8	0·30	8·6	12·6	2·9	2·6	46
609	7·0	2·3	4·7	0·5	0·6	0·30	10·9	11·5	2·3	2·6	41
828	7·0	3·1	3·9	0·8	1·0	0·30	9·4	12·5	2·5	2·8	58
1328	7·3	3·2	4·1	0·8	1·2	0·30	10·9	12·7	2·4	3·3	41
1335	7·2	3·4	3·8	0·9	1·1	0·30	10·3	13·2	2·6	2·9	46
1394	7·4	3·0	4·4	0·7	1·2	0·40	9·2	12·2	2·2	3·5	41
1418	7·1	3·2	3·9	0·8	1·2	0·35	8·1	12·8	2·3	2·2	41
1454	6·5	2·9	3·6	0·8	1·0	0·35	5·1	11·6	2·4	3·3	37
1470	7·0	2·9	4·1	0·7	0·9	0·30	10·7	12·5	2·0	3·1	29
1486	6·8	3·4	3·4	1·0	1·1	0·35	7·6	13·2	2·3	2·8	37
1492	7·3	3·2	4·1	0·8	1·1	0·30	7·1	12·6	2·5	2·6	41
1566	7·1	3·4	3·7	0·9	1·1	0·40	9·4	13·0	2·2	2·2	46
1576	6·8	2·8	4·0	0·7	0·9	0·20	8·5	12.2	2·2	3·3	33
1578	7·2	3·4	3·8	0·9	0·9	0·35	8·8	11·2	2·2	2·2	37
1614	7·5	3·3	4·2	0·8	0·9	0·20	7·0	13·3	2·5	3·0	41
1629	7·0	2·6	4·4	0·6	0·9	0·35	6·7	12·3	2·5	2·8	23
1724	7·3	2·7	4·6	0·6	1·1	0·30	7·8	12·5	2·7	3·2	32
1725	7·8	2·9	4·9	0·6	0·6	0·20	8·5	11.0	2·2	2·0	29
1794	7·5	3·6	3·9	0·9	0·9	0·35	12·0	13·1	2·6	2·4	41
1836	7·4	2·8	4·6	0·6	1·1	0·30	9·0	12·6	2·7	3·7	39
2605	7·7	3·4	4·3	0·8	1·1	0·35	7·0	11·9	2·1	2·9	37
6161	7·2	3·0	4·2	0·7	0·8	0·30	9·7	12·8	2·9	3·7	39
Mean	7·1	3·1	4·0	0·8	0·98	0·30	8·6	12·4	2·5	3·1	38·7
S. Deviation	±0·38	±0·34	±0·41	±0·14	±0·22	±0·06	±1·65	±0·58	±0·31	±0·79	±6·4
Range	6·0–7·8	2·3–3·8	3·2–4·9	0·5–1·2	0·6–1·2	0·20·–0·40	5·1–12·0	11·0–13·3	2·0–3·2	2·0–5·6	23–58

1950; Flynn and De Mayo, 1951; Levin and Oberholzer, 1952). The method is simple, the apparatus inexpensive and where results have been compared with those obtained with the Tiselius apparatus, agreement has been found to be good (Levin and Oberholzer, 1952.) The paper electrophoresis method described here for the measurement of the relative amounts of albumin and globulin would appear to be generally applicable to the sera of all domestic animals, and where information is required about the amounts of the individual globulins present, the paper strip may be divided into 5 mm. sections and the dye from each extracted and read as before (see Flyn and De Mayo, 1951). In this way one can construct an electrophoresis diagram as shown in Fig. 3. In the analysis of a single sample, the

Fig. 3

Electrophoretic diagram of normal horse serum, constructed by elution of dye from a 7 cm. strip as described in the text

TABLE 2

TOTAL PROTEIN, ALBUMIN AND GLOBULIN IN NORMAL HORSE SERA.

Author	*Year*	*Total protein g/100 ml.*	*Albumin g/100 ml.*	*Globulin g/100 ml.*	*A/G ratio*
Deutsch and Goodloe	1945	—	—	—	0·4
Svensson	1946	—	—	—	0·5*
Bonsmann and Weisner	1951	5·2 – 8·2	—	—	—
Westermarck	1942	7·1 ±0·45	—	—	—
Gilman	1952	6·3 – 8·6	2·3 – 3·5	4·0 – 5·1	0·55 – 1·0
Jennings and Mulligan	1953	6·0 – 7·8 (mean 7·1 ±0·38)	2·3 – 3·8 (mean 3·1 ±0·34)	3·2 – 4·9 (mean 4·0 ±0·41)	0·5 – 1·2 (mean 0·8 ±0·13)

* Calculated from the diagram given by Brooksby (1946) based on the results of Svensson (1946).

paper electrophoresis method is more time-consuming than a simple salting-out procedure, but this is more than offset by the fact that about 12 sera can be handled at the same time with the paper electrophoresis technique.

For the sake of comparison the results of the present investigations are shown in Table 2 along with those obtained by some other workers.

The values obtained for total protein in the present investigation are in good agreement with those reported by Westermarck (1942) and Gilman (1952). The range of albumin, globulin, and the A/G ratio agree closely with those of Gilman, but the mean A/G ratio is somewhat higher than the electrophoretic figure given by Deutsch and Goodloe (1945) and Svensson (1946). It should be noted, however, that the figures given by Deutsch and Goodloe were obtained on the analysis of sera from four aged horses at the time of slaughter. Gilman's figures were obtained by fractionation with 26 per cent sodium sulphate which is reported to give albumin values in good agreement with those obtained electrophoretically (Kilbrick and Blonstein, 1948) when applied to human serum.

Calcium, Magnesium and Inorganic Phosphate

The figures obtained in the present investigation are shown in Table 3 along with some other data from the literature. Agreement with other published figures is generally good.

TABLE 3

CALCIUM, MAGNESIUM AND INORGANIC PHOSPHATE IN NORMAL HORSE SERA

Author	*Year*	*Calcium mg./100 ml.*	*Magnesium mg./100 ml.*	*Inorganic phosphorus mg./100 ml.*
Eveleth	1937	—	2·0 – 3·0	—
Way	1942	11·0 – 13·8	—	2·0 – 5·0
Westermarck	1942	12·7 ± 0·13	—	3·1 ± 0·15
Sandstedt, Obel, Sjöberg and Karlsson	1946	10·6 – 13·6	—	2·0 – 5·9
Jennings and Mulligan	1953	11·0 – 13·3 (mean 12·4 ± 0·58)	2·0 – 3·2 (mean 2·5 ± 0·3)	2·0 – 5·6 (mean 3·1 ± 0·79)

Alkaline Phosphatase

There appears to be no published data on the level of serum alkaline phosphatase in the adult horse. Earle (1952) has studied the serum alkaline phosphatase in foals at various times between birth and six months of age. He observed a mean alkaline phosphatase activity equivalent to 109 ±9·2 'Bodansky'* units per 100 ml. serum at birth declining rapidly for the first 48 hours and then more slowly to a mean level of 17·3 ±0·9 'Bodansky' units at six months of age. That this decline continues with increasing age beyond six months is indicated by our mean figure of 8·6 ±1·65 'King-Armstrong' units per 100 ml. serum in the adult horse.

* The 'Bodansky' unit is one-third to one-half the King-Armstrong unit.

Bilirubin

Vilches (1949), using the Watson modification of the Malloy-Evelyn determination for bilirubin examined the plasmas of 100 normal horses. Average values obtained per 100 ml. were: total bilirubin, 0·975 mg.; direct bilirubin, 0·26 mg. Our figures: total bilirubin 0·98 ±0·28 mg./100 ml.; direct bilirubin 0.30 ±0.06 mg./100 ml. are in excellent agreement with those of Vilches. It is interesting to note that the normal level of serum bilirubin in the horse is significantly higher than that in man.

Urea

Dukes (1942) and Payne (1947) reports levels of blood urea N for the horse of 10 to 20 mg./100 ml. Our figures of 28 to 58 mg. urea/100 ml. serum, corresponding to a urea N range of 11 to 27 mg./100 ml. agree fairly closely with the earlier determinations, except that our upper limit is somewhat higher.

CONCLUSIONS

The sera of thirty normal horses have been analysed for total protein, albumin, globulin, calcium, magnesium, inorganic phosphate, alkaline phosphatase, bilirubin and urea. The results obtained are compared with other published data where available.

The technique of paper electrophoresis has been applied to the determination of albumin and globulin in horse serum and it is suggested that this may be a valuable routine method for studies on the serum proteins of domestic animals.

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THE USE OF ETHYLENE DIAMINE TETRA-ACETATE (EDTA) IN THE DETERMINATION OF CALCIUM AND MAGNESIUM IN URINE

By

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INTRODUCTION

The usual methods for estimating calcium and magnesium in urine, involving precipitations and washing of precipitates, are time consuming and tedious. The use of ethylene-diamine tetra-acetate for the determination of these ions in water suggested the possibility of extension of the more modern methods to urine.

Ethyl-diamine tetra-acetate (EDTA) chelates divalent ions such as magnesium and calcium. The reaction was first studied by Schwarzenbach, Biederman and Bangertes (1946) and by Bierderman and Schwarzenbach (1948) who showed that for each divalent ion chelated, 2 hydrogen ions are liberated. The release of hydrogen ions could be followed potentiometrically or with acid base indicators. They showed in addition that the dye Eriochrome Schwarz T. (Solochrome Black WFDA, ICI) will chelate magnesium ions, and also calcium ions provided magnesium is present, whereas ammonium purpurate (murexide) will chelate calcium ions only. They described the application of EDTA to the determination of calcium and magnesium hardness in water. These studies have been followed up by Connors (1950), Diehl, Goetz and Hoch (1950), Betz and Noll (1950), Heald, Coates and Edwards (1950) and the effect of various interfering ions determined. The application of these methods to the determination of calcium and magnesium in blood has been described by Sobel and Hanok (1951), Buckley, Gibson and Bartolotte (1951).

At pH 10 Eriochrome Schwarz T. chelates magnesium or calcium to give a wine red compound, while a metal-free solution of the dye is blue. Murexide at a pH > 11·5 in the absence of calcium ions is lilac, but when chelated it is rose pink. The chelates with EDTA are more stable than those with the dyes so that the dyes can be used to detect the removal of ions from the solution by EDTA.

The combined concentration of calcium and magnesium is determined by titration using the Eriochrome as an indicator. Using the murexide indicator the calcium is then determined and the magnesium found by difference. Alternatively, the calcium is precipitated with oxalate and the supernatant used for the direct titration of magnesium using the Eriochrome indicator.

This paper describes the applications of these methods to the determination of calcium and magnesium in urine. The original interest was in the urine of lactating cows containing little or no phosphate, but the extension of the method to human urine led to the adoption of certain modifications.

METHODS

Reagents

0·01M-EDTA. Dissolve 3·723 g. of disodium ethylene-diamine tetra-acetate ($Na_2C_{10}H_{14}O_8N_2.2H_2O$) in 500 ml. distilled water, bring the pH to 10 with N.NaOH and make up to 1 litre. This solution appears to be quite stable.

Calcium Indicator. Murexide is not very stable, particularly in aqueous solutions, and so the indicator is made by grinding 0·2 g. murexide with 100 g. of sodium chloride; in this form it keeps for a fortnight or more.

Magnesium plus calcium indicator. Dissolve 1 g. of Solochrome Black WFDA (ICI Ltd.) in 25 ml. of distilled water; after adding 1 ml. of $N.Na_2CO_3$ make up to 100 ml. with *iso*-propyl alcohol, filter through a Buchner funnel and keep at 4°C.

Ammonia Buffer. Dissolve 67·5 g. ammonium chloride in 570 ml. of concentrated ammonia, and make up to 1 litre with distilled water.

Sodium hydroxide	10 N.
Potassium Cyanide	0·7 M.
Sodium Chloride	0·1 M.

Methods

Two methods are available—

Bovine urine. (Containing little phosphate). To each 100 ml. of urine, 1 ml. concentrated nitric acid is added to dissolve the carbonate and to bring the pH value between 3 and 4 before filtering. Fifty ml. of filtered urine are diluted to 100 ml. with 0·1M-NaCl and 10 ml. aliquots of this solution are used for the titrations. For the determination of calcium, about 40 mg. of calcium indicator from a spoon. 1 ml. of 0·7M.KCN and 1 ml. 10N.NaOH are added. The colour should be rose pink. EDTA solution is run in with vigorous shaking until the colour starts to change towards lilac; after this the titration should proceed slowly (30 second intervals between additions) until there is no further change towards lilac. It is an advantage to use for comparison a similar aliquot containing an excess of EDTA.

The total of calcium plus magnesium is determined in another aliquot to which is added 2 drops of magnesium indicator, 1 ml. ·7M cyanide and 2 ml. of ammonia buffer. In this case the colour change is from wine red to green or blue depending on the amount of yellow pigment in the urine.

The concentration of calcium or magnesium plus calcium ions in filtered urine is given by

$$Y \times \cdot 01 \times F \times \frac{100}{10} \times \frac{1000}{50} = Y \times 2F \text{ mg. mol/l}$$

where Y is the volume of ·01M EDTA solution used for a 10 ml. aliquot of diluted urine and F is the factor for this solution. The magnesium concentration is the difference between the combined

TABLE III

RECOVERY OF CALCIUM AND MAGNESIUM ADDED DURING PROCESS OF DILUTION

Sample	No. of Observations	Original Conc. Present			Amount Added		Amount Found			Percentage Recovery		
		Total mg. mol/l	Ca.++ mg. mol/l	Mg.++ mg. mol/l	Ca.++ mg. mol/l	Mg.++ mg. mol/l	Total mg. mol/l	Ca.++ mg. mol/l	Mg.++ mg. mol/l	Total	Ca.++	Mg.++
Cow B ..	3	24·49	10·09	14·39	4·14	1·76	30·37	14·14	16·26	99·7	99	101·3
Cow F ..	2	13·85	3·41	10·44	1·5	1·60	16·95	4·93	12·02	100·0	101·3	98·8
Cow G ..	2	14·5	1·81	12·69	1·5	1·6	17·54	3·26	14·28	98·4	96·7	99·4
Man X ..	2	11·1	6·28	4·72	2·5	2·06	15·74	8·78	6·96	100·5	100·0	102·6
Man Y ..	2	1·46	·92	·54	2·5	2·06	5·98	3·36	2·63	99·1	98·2	101·1
Average % Recovery										99·54	99·04	100·6

All entries are the average of the number of observations shown. Method I used for bovine and Method II for human urine.

concentration and the calcium concentration. The factor F is determined by standardising the EDTA solution against a known mixture of calcium and magnesium.

Human urine. When the above method is applied to human urine containing phosphate the addition of murexide produces the colour of the metal-free solution. To overcome this difficulty the filtrate from a uranyl acetate determination of phosphate is used. Twenty ml. of urine are taken and the phosphate titrated with standard uranyl acetate, made up to 50 ml. with 0.1M. NaCl and filtered. The filtrate is treated in the same way as the diluted urine in method 1. In order to get a sharp endpoint it may be necessary to add more Solochrome indicator.

The concentration of calcium or magnesium plus calcium ions is given by

$$Y \times \cdot 01 \times F \times \frac{50}{10} \times \frac{1000}{20} = Y \times 2{\cdot}5\ F \text{ mg. mol/l}$$

the symbols having the same meaning as the calculation in method 1 and the magnesium determined by difference.

In the event of only small amounts of urine being available, both titrations can be done on one aliquot of diluted urine. In this case, following the calcium titration, acid is added to reduce the pH to less than 3. This destroys the murexide, leaving the complex of calcium and EDTA. When all the murexide has been destroyed, solochrome indicator is added and the magnesium titrated.

RESULTS

Tables I and II show the results obtained using these methods on a number of bovine and human urines. Method 1 was used for the bovine and method 2 for the human urines. For method 1 the difference between replicate determinations is usually about 2 per cent or less while for method 2 it is usually 5 per cent or less.

TABLE I

DETERMINATION OF URINARY CALCIUM AND MAGNESIUM IN BOVINE URINE USING METHOD 1.

Cow	*No. of observations*	*"Total" Conc. mg.mol/l Ca++ plus Mg++*			*Calcium Conc. mg.mol/l Ca++*			*Magnesium Conc. mg.mol/l Mg++*		
		Mean	*Range * Actual*	*% mean*	*Mean*	*Range * Actual*	*% mean*	*Mean*	*Range* Actual*	*% mean*
A	2	11·72	0·1	0·85	0·40	0·00	0·0	11·33	0·03	0·27
B	3	24·49	0·2	0·82	10·09	0·10	1·0	14·39	0·1	0·69
C	2	8·70	0·02	0·23	2·63	0·06	2·3	6·07	0·08	1·31
D	2	15·49	0·09	0·58	3·13	0·00	0·0	12·36	0.10	0·80
E	2	20·01	0·04	0·20	7·36	0·13	1·8	12·65	0·10	0·79
F	2	13·85	0·04	0·29	3·41	0·02	0·59	10·44	0·06	0·57
G	2	14·50	0·08	0·55	1·81	0·02	1·10	12·69	0·06	0·47
Mean Range		—	0·081	0·503	—	0·047	0·97		0·076	0·70

*Range is the difference between highest and lowest value observed for that sample, i.e. the sample range.

TABLE II

DETERMINATION OF CALCIUM AND MAGNESIUM IN HUMAN URINE USING METHOD 2

Man	No. of observations	"Total" Conc. mg.mol/l Ca++ plus Mg++			Calcium Conc. mg. mol/l Ca++			Magnesium Conc. mg. mol/l Mg++		
		Mean	Range Actual	% mean	Mean	Range Actual	% mean	Mean	Range Actual	% mean
V	2	15·09	0·1	0·663	8·74	0·10	1·144	6·35	0·12	1·89
W	2	8·12	0·12	1·478	2·69	0·05	1·859	5·43	0·17	3·13
X	2	11·10	0·00	0·00	6·28	0·12	1·911	4·72	0·12	2·54
Y	2	3·24	0·12	1·478	1·16	0·02	1·724	2·09	0·13	6·22
Mean Range		—	0·085	0·905	—	0·073	1·66		0·155	3·45

Table III shows the results of a number of recovery tests, the figures given being means of at least two determinations. By both methods recovery is between 98 and 102 per cent.

DISCUSSION

The most important advantage of the method described is its speed and simplicity as compared with the usual methods involving precipitation. A little experience is needed in observing the colour change of the indicators and it is recommended that a sample containing an excess of EDTA be used for comparison.

Buckley et al (1951) recommend the use of cyanide to prevent interference by heavy metals in serum but in urine the principal interference is likely to be due to phosphate. This difficulty can be overcome by using the filtrate from the uranyl acetate determination of urinary phosphate.

With samples containing very small amounts of calcium and magnesium the preliminary dilution with 0·1M NaCl need not be performed.

SUMMARY

A rapid titrimetric method for the determination of calcium and magnesium is described. The method is accurate to within 5 per cent.

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LAMINAR FLOW IN THE PORTAL VEIN OF THE SHEEP AND GOAT

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INTRODUCTION

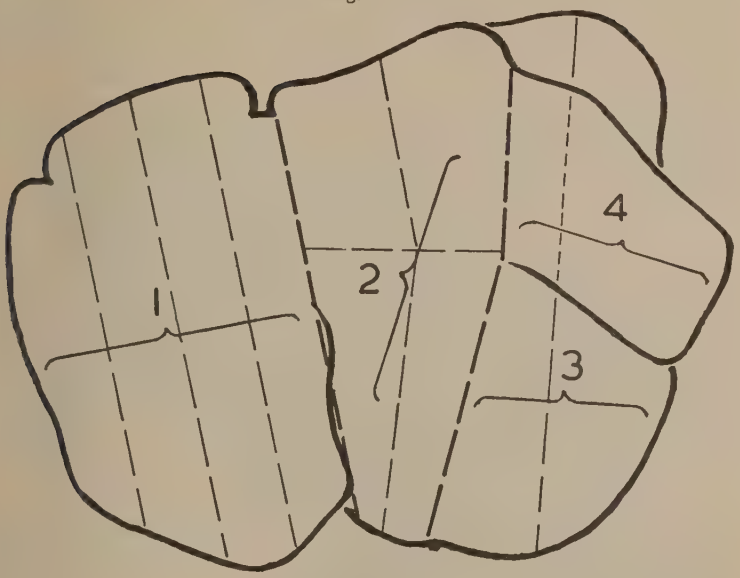
Aspiration biopsy of the liver in the bovine leads to the removal of a small sample of tissue from the extreme right edge of the liver (Garner, 1950; Loosmore and Allcroft, 1951). The occurrence of 'stream-line' flow, leading to uneven distribution of portal blood to the liver, as first suggested by Séregé (1902), has been directly demonstrated in the dog (Copher and Dick, 1928; Hahn, Donald and Crier, 1945) and in the rat (Himsworth, 1947), and can be inferred in man from the distribution of the lesions in dietetic necrosis. Such a bilateral flow in the bovine could lead to a similarly localized distribution of hepatic lesions; a biopsy specimen might then be unrepresentative of the whole organ and hence of doubtful diagnostic value. Using colloidal chromic phosphate labelled with P^{32}, it has been found that laminar flow can indeed be demonstrated in the portal vein of the sheep and goat, but that the distribution of portal blood does not follow the constant pattern observed in other species.

METHODS

The results reported were obtained in four adult sheep and five adult goats.

Chromic phosphate was prepared from radioactive phosphoric acid by a procedure based on that of Jones, Wrobel and Lyons (1944). 300mg. of the anhydrous powder (containing approximately 200μc of P^{32}) was suspended in 4-8ml. of isotonic glucose solution by shaking with ballotini (Grade 12) in an oscillating shaker. The colloidal suspension thus obtained was filtered through glass wool and injected into the spleen of the anaesthetized animal. The liver was immediately removed and divided into two in a plane parallel to the surfaces. One part was cut into narrow strips approximately parallel to the plane of separation of the right and left lobes (Fig. 1); representative portions of tissue taken from these strips were weighed and wet-ashed using sulphuric and nitric acids. After neutralization, suitable aliquots were taken for determination of P^{32}. The remaining portion of the liver was fixed in 10 per cent formol-saline, a slice about 5mm. thick cut from it and this section placed on Kodirex 'Blue-brand' X-ray film with two thicknesses of copper foil (density 128mg./cm^2.) interspersed and left for 72 hours. The film was developed in Kodak D-19b X-ray developer.

Fig. 1



RESULTS

The distribution of the isotope is given in Table I. The data have been simplified by averaging the results for the various sections grouped together as indicated in Fig. 1. The autoradiographs gave

TABLE 1

DISTRIBUTION OF P^{32} IN LIVERS

		*Thousands of counts per minute per g. wet weight of tissue Section**			
		1	2	3	4
Sheep No.	1	16	16	18	19
	2	25	34	28	25
	3	34	21	25	24
	4	28	36	43	37
Goat No.	1	32	30	16	10
	2	20	25	46	221
	3	24	35	52	61
	4	63	49	26	37
	5	37	14	40	27

*See Fig. 1 for explanation of sections.

results in close agreement with the counts. In six animals there was an obvious difference in the distribution of P^{32} across the liver; in one sheep and two goats (the distribution in one goat is shown in Fig. 2) the isotope was concentrated towards the left side and in

another sheep (Fig. 3) and a further two goats towards the right side. Of the remaining animals, one goat showed concentration on the right side, but some chromic phosphate appeared to have reached the extreme left border, the middle being relatively clear (Fig. 4), while the two sheep showed an almost even distribution over the whole liver. In all animals in which partition occurred, the line of demarcation corresponded to the line of attachment of the gall-bladder and cystic duct.

DISCUSSION

These results show that laminar flow can occur in the portal vein of the sheep and goat and that the right and left branches of the portal vein supply quite distinct areas of the liver. The partition of portal blood follows exactly the distribution which would be expected were the branches of the portal vein divided along a line from the gall-bladder fossa to the point of entrance of the hepatic veins into the inferior vena cava, as described in man (McIndoe and Counsellor, 1927). Further evidence that such a partition occurs in the calf can be adduced from the distribution of hepatic abscesses following omphalophlebitis (Frei, 1936).

In other species so far examined it has been found that splenic blood always proceeds *via* the left branch of the portal vein to the left lobe(s) of the liver. In sheep and goats it seems possible that blood from the spleen may flow either to the right or left. It is suggested that an alternation of flow might occur as a result of alterations in the relative positions of the mesenteric and gastro-splenic tributaries of the portal vein as a result of variations in the degree of distension of the rumen. Dissection of *post-mortem* specimens supports the view that the portal vein may be fixed at its hepatic extremity and yet relatively free to rotate at its distal end.

Should blood from the spleen (and hence also from the intestine) alternate between the left and right lobes of the liver in the living animal, as the results suggest, a biopsy specimen from any part of the liver could be taken as fairly representative of the whole organ.

Corrosion specimens obtained by injecting 'Neoprene' (B. B. Chemical Co.) into the portal vein *in situ* show that the bifurcation of this vessel into its hepatic branches takes the form of a 'T', the portal vein and its right branch being represented by the horizontal arms of the T and the left branch by the vertical stroke. Julian and De-Ome (1949), in describing the bovine liver, give diagrams in which the bifurcation appears to be Y-shaped. This is not true of the sheep and goat.

SUMMARY

By following the fate of radioactive chromic phosphate injected into the spleen, it has been demonstrated that laminar flow can occur in the portal vein of the sheep and goat.

FIG. 2

To face page 302

FIG. 3

FIG. 4

In these species splenic blood may be diverted to either the right or left lobes of the liver. Possible reasons for this phenomenon are discussed.

ACKNOWLEDGMENTS

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LEGENDS TO FIGURES

Fig. 1. Diagram showing method of sectioning liver for isotope counts. The figures refer to the grouping of the sections employed in compiling Table 1. Visceral surface of liver shown.

Figs. 2 and 4. Autoradiographs of goat livers, following injection of radioactive chromic phosphate into the spleen.

Fig. 3. Autoradiograph of sheep liver.

EXPERIMENTAL STREPTOCOCCAL MASTITIS

VIII. Further Immunological Studies in Goats

By

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INTRODUCTION

As a preliminary experiment to investigate the possibility of protecting the mammary gland by the use of vaccines, Holman, Pattison and Gordon (1952) subjected goats to repeated intraperitoneal injections of broth cultures of living *Str. agalactiae* strain S 13. Following an intramammary injection of a test dose of the same organism, measurable protection of the udder was demonstrated.

As the use of a living vaccine against an intramammary test dose of the homologous organism had thus proved successful, and as earlier work by Pattison (1948) had shown that dead vaccines could produce in mice active immunity that was apparently type-specific, it was decided to test the protective value of a dead, polyvalent vaccine against experimental mastitis in goats.

This paper records the results of experiments in mice, giving further proof that with group-B streptococci both active and passive immunity were, in part at least, type-specific; and in goats, showing that parenteral injection of a polyvalent formolised vaccine of group-B streptococci gave measurable protection against an intramammary test dose of the different individual type strains present in the vaccine.

MATERIALS AND METHODS

Experimental animals. The goats used were two to five years old, housed indoors and had not previously been used for any experiment. Streptococci were not isolated from the milk of any animal prior to experimental infection.

Strains of group-B streptococci. Through the kindness of Dr. R. C. Lancefield of the Rockefeller Institute, New York, we were in possession of her four types identified by the precipitin test (Lancefield, 1938). These were :— Type Ia, strain O 90; type Ib, strain H 36 B; type II, strain 18 RS 21; type III, strain, M 216. These four strains, together with our strain S 13, that had been classified by Lancefield as belonging to type Ib, were the vaccine and test strains used.

Preparation of vaccines for use in mice and goats. Vaccines were freshly prepared each week by growing individual type strains in Todd-Hewitt broth for 18 hours, plating for purity, and adding 0·3 per cent formalin. All vaccines were non-viable 24 hours after the addition of formalin. Polyvalent vaccines were made by pooling equal volumes of the appropriate strains.

Active immunisation of mice. Mice received eight intraperitoneal injections of 0·5 ml. of vaccine at three day intervals, and were then exposed to a 0·5 ml. intraperitoneal test dose of organisms grown for 18 hours in Todd-Hewitt broth. Dilutions of organisms for the various test doses were made in saline and were injected within 15 minutes of the dilution being made. Deaths were counted after three days.

Passive immunisation of mice. Sera for passive protection tests in mice were made by injecting goats subcutaneously with ten 20 ml. doses of vaccine over a period of 60 days. Serum was drawn three months after the last immunising dose. Mice were given an intraperitoneal injection of 0·5 ml. of the serum to be tested, followed next day by a test dose as described under active immunisation.

Active immunisation of goats and exposure to the intramammary test dose. Four intraperitoneal, or subcutaneous injections of 10 ml. of vaccine were given at intervals of one week (see Table page 310); the goats were then rested for three weeks after which they received two more weekly injections. Twelve days later a test dose of 1·0 ml. of an 18-hour Todd-Hewitt broth culture of the appropriate strain, brought to a volume of 10 ml. by the addition of sterile Todd-Hewitt broth, was injected through a smooth-ended needle into the teat canal of one side of the udder.

Measurement of the degree of mastitis caused by intramammary inoculation of the test dose. Measurements were limited to those tests we have found in previous experiments to give the most reliable indication of dysfunction of the mammary gland, namely milk yield and neutrophil and bacterial content of the milk. Yield of milk was measured twice daily and the average for one week for the infected side was recorded as a percentage of the amount predicted for that side by the yield of the non-infected side (Holman, Pattison and Gordon, 1952). Neutrophil and bacterial counts were carried out once each week. Neutrophils were counted as described by Pattison and Holman (1951), and the technique for making bacterial counts was that used by Pattison, Taylor and Holman (1950).

Statistical analysis of data. In an effort to obtain an objective assessment of the figures given in Tables 7 and 8, use was made of the variance ratio method described by Mainland (1938) and Yule and Kendall (1946). The use of this test appeared to be justified in that the various types in both groups of controls were quite homogeneous, suggesting that the data were sufficiently normal for the test to be valid.

Histological methods. The methods used for fixing, embedding, and staining goat mammary tissue have been described by Pattison (1951).

RESULTS

Experiments with Mice

Examination of the mouse-lethal power of Lancefield's type strains of group-B streptococci. As a preliminary to immunity experiments in mice it was necessary to determine the mouse-lethal power of Lancefield's strains. The results of various experiments are summarised in Table 1 and show that 0 90 had considerable killing power even in high dilution, as observed by Lancefield (1934), that H 36 B and 18 RS 21 were sufficiently lethal to be used, but

TABLE 1

RESULTS OF INTRAPERITONEAL INOCULATION WITH 0·5 ml. TEST DOSES

(Various dilutions of Lancefield's type strains of group-B streptococci grown for 18-hours in Todd-Hewitt broth of (a) untreated mice (b) mice inoculated intraperitoneally 18 hours previously with 0·5 ml. of non-immune serum.)

Serological type	*Strain*	*Deaths in untreated mice*					*Deaths in mice inoculated i.p. 18 hours previously with 0·5 ml. non-immune serum*			
		Undiluted	10^1	10^2	10^3 & 10^4	10^5 & 10^6	*Undiluted*	10^1	10^4	10^5 & 10^6
Ia	o 90	60/60 =100%	20/20 =100%	25/25 =100%	94/95 =99%	217/233 =93%	—	—	69/70 =99%	67/70 =96%
Ib	H 36 B	138/140 =99%	126/134 =94%	70/132 =53%	—	—	79/80 =99%	60/80 =75%	—	— —
II	18 RS 21	152/158 =96%	51/132 =39%	1/10 =10%	—	—	92/100 =92%	—	—	—
III	M 216	Pathogenicity very low: untreated mice— 0·5 ml. 1 death out of 20: 1·0 ml. 1 death out of 10: 1·5 ml. 5 deaths out of 30.								

— = not tested

that strain M 216 had so little killing power that it was of no value as a test strain.

In the passive immunity tests described below, test doses for control mice were preceded by injection of non-immune serum, and the figures in Table 1 suggested that in smaller doses this procedure had slightly lowered the death rate. To clarify this point some of the figures have been further analysed in Table 2 and it can be seen

TABLE 2

FREQUENCY DISTRIBUTION OF MICE

(Surviving after (i) Test dose alone (ii) Intraperitoneal injection of 0·5 ml. of non-immune serum followed the next day by the test dose.)

Strain	*Dilution*	*Group*	*Number of tests*	*Distribution of survival rates for tests on 10 mice*						
				0	1	2	3	4	5	6
0 90	10^{-6}	Test dose alone	9	3	4	1		1		
		Serum then test dose	2		1	1				
H 36B	10^{-1}	Test dose alone	13	8	3		2			
		Serum then test dose	8		3	2	1	1		1
18 RS21	10^{0}	Test dose alone	14	11	3					
		Serum then test dose	10	3	4	2	1			
Totals for all tests (telescoped to give 4-cell test)										
		Test dose alone	36		32	4	(X^2=8·26			
		Serum then test dose	20		11	9	(p =·01/·001			

that it was unlikely that this lowered death rate had been due to chance alone. A similar phenomenon by which the injection of killed vaccines, sterile broth etc. into the peritoneal cavity decreased the lethal effect of a subsequent test dose has been recorded by Felix (1951) working with Salmonella, by Pfeiffer and Issaeff (1894) working with *V. cholerae*, and has been noted by Coid (1953) working in this laboratory with *Br. abortus*. In spite of this drawback to the use of the intraperitoneal route for both serum (or vaccine) and test dose in mouse protection tests, the simplicity of the method has led us to continue using it.

Type specificity of active immunity in mice. Numerous experiments were carried out to examine the specificity of active immunity to Lancefield's type strains. The results of two of these experiments, typical of them all, are recorded in Tables 3 and 4.

In Table 3 the test doses used were strain 0 90 at 10^{-4} dilution, and strains H 36 B and 18 RS 21 undiluted. It will be seen that the mixed vaccine gave good protection and that vaccines for types

TABLE 3

RESULTS OF EXPOSING TO LARGE INTRAPERITONEAL TEST DOSES

(Lancefield's type strains (a) mice previously actively immunised by intraperitoneal injection of vaccine for these type strains (b) untreated mice.)

Mice actively immunised against type	*No. of mice*	*Survivors after test with Type*		
		Ia	*Ib*	*II*
Strain		O 90	H36B	18RS21
Dilution		10^{-4}	*Undil.*	*Undil.*
Dose		0·5 ml.	0·5 ml.	0·5 ml.
Ia (O 90)	11	11	0	5
Ib (H 36B)	11	2	9	1
II (18RS21)	11	2	0	11
III (M216)	11	3	1	6
Ia + Ib + II + III ..	11	11	10	11
Control: expected survivors among untreated mice see Table 1	11	0·01	0·01	0·04

Ia, Ib, and II showed clear evidence of type-specific protection. However, a striking finding was that each vaccine (including type III, for which no test strain of the same type was available) gave some degree of non-specific protection by comparison with the untreated control mice. It could be argued that this non-specific protection was given by the group antigen, but in view of the phen-

TABLE 4

RESULTS OF EXPOSING TO A SMALL INTRAPERITONEAL TEST DOSES

(Lancefield's type strains (a) mice previously actively immunised by intraperitoneal injection of vaccine for these type strains (b) untreated mice)

Mice actively immunised against type	*No. of mice*	*Survivors after test with type*		
		Ia	*Ib*	*II*
Strain		O 90	H36B	18RS21
Dilution		10^{-6}	10^{-2}	10^{-1}
Dose		0·5 *ml.*	0·5 *ml.*	0·5 *ml.*
Ia (O 90)	11	11	9	11
Ib (H36B)	11	1	11	11
II (18RS21)	11	3	7	11
III (M 216)	11	7	7	11
Ia + Ib + II + III ..	11	11	11	11
Control: expected survivors among untreated mice, see Table 1	11	0·07	4·7	6·1

omenon, already discussed, in which the lethal power of an intraperitoneal test dose is reduced if previous intraperitoneal injections have been given, we feel that the eight intraperitoneal injections of foreign protein material had probably given the mice an entirely non-specific protection against a test dose by the intraperitoneal route.

The existence of some non-specific protection seemed even more likely when mice were exposed to less lethal test doses, as recorded

in Table 4. It will be noted that every figure for the intraperitoneally vaccinated mice is greater than the expected number of survivors among untreated mice.

The type specificity of passive immunity in mice. In view of the difficulty of interpreting results in active immunity experiments, it was considered important to examine the specificity of passive immunity in mice produced by injection of serum prepared in goats against Lancefield's type strains. For this experiment the test doses of the type strains were the same as those in Table 3. The control mice received non-immune serum and the expected death rate for these animals is given in Table 1.

Results of this test are given in Table 5 and showed clear evidence of type-specific immunity. Immunity to types Ia and II

TABLE 5

RESULTS OF EXPOSING TO LARGE INTRAPERITONEAL TEST DOSES

(Lancefield's type strains (a) mice previously passively immunised by intraperitoneal injection of immune serum for these type strains (b) mice previously injected intraperitoneally with non-immune serum.)

Mice passively immunised against type	*No. of mice*	*Survivors after test with type*		
		Ia	*Ib*	*II*
	Dilution Dose	10^{-4} 0·5 *ml.*	*Undil.* 0·5 *ml.*	*Undil.* 0·5 *ml.*
Ia (O 90)	10	10	0	3
Ib (H36B)	10	0	4	1
II (18RS21)	10	0	0	9
III (M 216)	10	0	0	0
Ia + Ib + II + III ..	10	10	3	3
Control: expected survivors after treatment with non-immune serum, see Table 1	10	0·01	0·01	0·08

was good, but to Ib was only moderate. Type III serum gave no protection at all. Serum from a goat injected with polyvalent vaccine gave good protection against type Ia, slight against Ib, and apparently none against II.

The interpretation of the results of active and passive immunity tests in mice. Both active and passive immunity tests in mice can be greatly influenced by three variable factors, none of which is adequately controlled in the type of experiment detailed above :—

(a) Strains of streptococci of the same serological type vary greatly in their mouse-lethal power.

(*b*) Strains of streptococci may vary in their ability to stimulate antibody production.

(c) Mice vary in their susceptibility to the lethal action of streptococci.

(d) Goats receiving the same polyvalent vaccine show a big difference in the amount of mouse-protecting antibody they produce to the different types.

On these circumstances the interpretation of results must be cautious and emphasis should not be laid on minor differences in figures.

Bearing these facts in mind, however, we consider that the experiments detailed above indicate clearly the presence of some degree of type specificity in immunity to group-B streptococci.

Experiments with Goats

Plan of experiment. The results of experiments with mice, reported above, confirmed that immunity was in part at least type specific and that a vaccine to protect goats against intramammary test doses of group-B streptococci of Lancefield's four types should contain organisms of all these types.

In previous experiments (Holman, Pattison and Gordon, 1952) it was shown that detectable antibody could be produced in goats by injection of living strain S 13 by a variety of routes (subcutaneous, intravenous, intradermal, intraperitoneal), and that no one route appeared to produce a significantly greater amount of antibody than another. Experiments with mice indicated that formolised culture was not less efficient than living culture in producing protective antibody. In view of these findings it was decided to use formolised whole cultures as vaccines, and to inject them subcutaneously into some animals and intraperitoneally into others. A polyvalent vaccine containing strains O 90, 18 RS 21, H 36 B and M 216 was used for all goats. They were then exposed to an intramammary test dose of one strain only. The distribution of the 25 animals in the experiment was as follows :—

	Number of goats exposed to an intramammary test dose of strain :—				
	O 90	H 36 B	18 RS 21	M 216	S 13
Vaccinated subcutaneously ..	1	1	1	1	1
Vaccinated intraperitoneally ..	2	2	2	2	2
Non-vaccinated	2	2	2	2	2

A group of goats exposed to a test dose of strain S 13 (type Ib) was included because previous studies on reactions to intramammary inoculation has been limited to this strain, and it was thought that reactions in this group of animals would serve as a standard by which reactions to the other strains might be judged.

Examination of sera from vaccinated and non-vaccinated goats for mouse-protecting antibody. Six days after the final injection of vaccine all the 15 vaccinated animals and the ten animals selected as controls were bled and their sera tested for mouse-protecting antibody. Sera were sterilised by filtration before use.

The results of these tests are given in Table 6, and it will be seen that there was a highly significant difference between the vaccinated animals as a group and the non-vaccinated as a group. The serum

TABLE

RESULTS OF MOUSE-PROTECTION TESTS (EACH TEST WITH FIVE MICE) ON SERA FROM 15 VACCINATED AND 10 NON-VACCINATED GOATS

Group	*No. of goats*	Frequencies of deaths out of 5 mice previously inoculated with serum from vaccinated or non-vaccinated goats and now exposed to a test dose of strain :—																			
		O 90 *Tested at* 10^5 *&* 10^4							*H*36*B* *Tested undil. and* 10^1							18*RS*21 *Tested undiluted only*						
		No. of tests	*Deaths out of 5 mice*						*No. of tests*	*Deaths out of 5 mice*						*No. of tests*	*Deaths out of 5 mice*					
			0	1	2	3	4	5		0	1	2	3	4	5		0	1	2	3	4	5
Vaccinated	15	30	10	8	3	3	3	3	30	15	4	1	4	2	4	15	4	0	2	2	4	3
Non-vaccinated	10	20	0	0	0	0	1	19	20	0	0	0	2	4	14	10	0	0	0	0	2	8

of some vaccinated goats, however, gave no evidence of the presence of mouse-protecting antibody against one or more of the test strains; if deaths of either four or five mice out of five be taken as indicating the absence of antibody, then six tests of sera from vaccinated animals showed no antibody against strain O 90, six tests showed none against strain H 36 B and seven tests showed none against strain 18 RS 21.

Examination of the complete data showed that the vaccinated goats could be grouped according to the mouse-protecting value of their serum as follows :—

(*a*) Good protection against three test strains—four animals (three injected intraperitoneally and one subcutaneously)

(b) Good protection against two test strains—five animals (three injected intraperitoneally and two subcutaneously)

(c) Good protection against one test strain—five animals (four injected intraperitoneally and one subcutaneously)

(d) No protection against any test strain—one animal (injected subcutaneously)

It was therefore concluded that the vaccine caused varying degrees of antibody response in different individuals, and that in some animals the amount of detectable antibody to the test strains used was markedly different. There was no obvious difference between the amount of mouse-protecting antibody produced by intraperitoneal and by subcutaneous injection of the vaccine; animals with well developed or with poorly developed antibody occurred in both groups.

The correlation between amount of mouse-protecting antibody and the degree of protection shown against mastitis in the goat was slight and uncertain and similar to that previously shown in experiments with strain S 13 (Holman, Pattison and Gordon, 1952).

Results of intramammary infection of vaccinated and non-vaccinated goats. The results of exposing 15 vaccinated and ten non-vaccinated goats to an intramammary test dose of group-B streptococci are recorded in Figure 1 and Tables 7 and 8. In assessing these results the milk yield has again been regarded as the most important criterion of mastitis; the yield of the infected gland expressed as a percentage of the expected yield (as estimated from the non-infected gland) is shown in Figure 1. If a deficiency of more than 50 per cent of the estimated yield of milk is accepted as an arbitrary figure for classifying a definite clinical mastitis, it can be seen that after the first week immunised goats in all groups were above this standard and control goats in three groups were below it. The results for control animals inoculated with strains in 18 RS 21 and M 216 (types II and III) were above the 50 per cent standard, and this suggests that these two strains did not cause obvious mastitis

Fig. 1

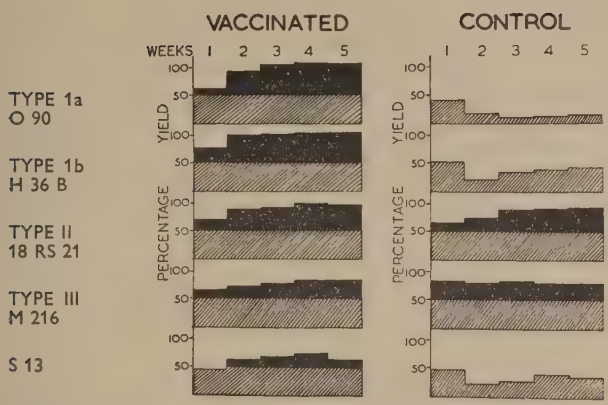

Fig. 1. Showing average loss of milk from the inoculated quarters of each group as compared to the amount predicted for these quarters from the yield of the control glands. Amounts over 50 per cent. are blacked in.

TABLE 7

MEANS AND DISTRIBUTIONS OF NEUTROPHIL LEUCOCYTES

(Per ml. of milk in five successive weekly tests made on three vaccinated and two control goats in each group following the test dose.)

Strains used to test goats immunised by a polyvalent vaccine		*Neutrophils, in millions per ml. of milk*					*Variance ratio value of p.*
		Mean	*Classes and midpoints*				
			0 to 0·3 (0·15)	0·3 to 1·0 (0·65)	1·0 to 3·0 (2·00)	over 3·0 (15·00)	
O 90	Vaccinated	0·09	12	2	0	1	<·002
	Controls	27·12	2	2	1	5	
H36b	Vaccinated	0·07	12	3	0	0	<·002
	Controls	2·08	1	0	5	4	
18RS21	Vaccinated	0·09	10	3	2	0	<·002
	Controls	1·79	4	2	1	3	
M216	Vaccinated	1·50	6	0	4	5	>·400
	Controls	0·85	3	3	2	2	
S13	Vaccinated	0·75	6	3	2	4	·001*
	Controls	8·20	1	0	1	8	

*Analysis of variance

even in non-vaccinated animals; proof of the presence of some degree of immunity among vaccinated animals in these groups had therefore to depend on other measurements.

In previous experiments a high neutrophil count in the milk has been accepted as a good secondary measurement of the degree of mastitis. The distribution of neutrophil counts for vaccinated and non-vaccinated goats are recorded in Table 7. These figures show that in all the immunised groups there was a bias towards low counts, and that in all groups except that inoculated with strain M 216 the difference between immunised animals and their controls was unlikely to be due to chance alone.

Table 8 records the distribution of bacterial counts of milk samples taken over the same period. Here again the bias in all the

TABLE 8

MEANS AND DISTRIBUTIONS FOR NUMBERS OF *STR. AGALACTIAE*

(Per ml. of milk in five successive weekly tests made on three vaccinated and two control goats in each group following the test dose.)

Strain used to test goats immunised by a polyvalent vaccine		*Organisms per ml. of milk mid-points of class intervals*				*Variance ratio value of p.*
		Mean	0	10,000	50,000	
O 90	vaccinated	875	8	7	0	<·002
	controls	27,220	1	5	4	
H 36b	vaccinated	104	12	3	0	<·002
	controls	8,470	0	6	4	
18RS21	vaccinated	12,130	5	9	1	>·100
	controls	12,500	0	7	3	
M 216	vaccinated	8,280	0	15	0	<·002
	controls	25,300	0	7	3	
S 13	vaccinated	8,790	0	15	0	<·002
	controls	45,000	0	3	7	

immunised groups was towards low numbers, and it can be seen that with the exception of the group inoculated with strain 18 RS 21, this bias was unlikely to be due to chance alone.

It can be said, therefore, that with the three strains, o 90 (type Ia), H 36 B (type Ib), and S 13 (type Ib), that produced marked clinical mastitis there was a significant difference in favour of the immunised groups in all three measurements. With strain 18 RS 21 (type II) alteration in the milk yield of the control goats indicated little evidence of mastitis, but the neutrophil reaction was significantly less in the immunised group. With strain M216 (Type III) the milk yield for the control goats exceeded that of the immunised goats. This was due to a severe fall in milk yield in one of the immunised goats, so that the immunised goats gave 96, 90 and 42 per cent

of the predicted yields, as compared to the two control goats that produced 90 and 71 per cent. The average milk leucocyte count in the immunised goats was in each case lower than those for the control goats and the distribution of the bacterial counts was significantly lower than in the controls.

It is evident, therefore, that the polyvalent vaccine produced a substantial protection of the mammary gland against infection by each of the type strains of group-B streptococci, but that the result was not as clear cut as could have been wished owing to the failure of the strains representing types II and III to cause definite, easily measurable, clinical mastitis.

Histological examination of the udders of control animals. In previous experiments reported in this series all intramammary inoculations for the experimental production of mastitis by group-B streptococci had been of *Str. agalactiae* strain S 13, and histological examinations had therefore been limited to the study of tissue reactions to this organism. The use of Lancefield's type strains in these experiments, however, provided an opportunity for examining mammary glands inoculated with strains of group-B streptococci other than S 13. The ten control animals in these experiments were therefore killed and their udders removed for histological examination.

Examination of these ten udders showed that the disease produced by Lancefield's type strains was in no way different from that produced by S 13. Extensive damage, comparable to that seen in marked reactions to inoculation of S 13, was caused in both animals inoculated with o 90 and in one animal inoculated with H 36 B. Less extensive damage, but with the disease quite clearly progressive, was found in the second animal inoculated with H 36 B. The udders inoculated with 18 RS 21 and M 216 showed the same picture as in minimal reactions to inoculation of S 13, namely an extensive increase in interacinar cellularity but without evidence of streptococcal penetration from the duct system to the interacinar tissue, and without significant damage to the secretory epithelium.

DISCUSSION

All Lancefield's type strains grew and persisted in the udder of the control goats over the five week experimental period, and the mastitis produced was indistinguishable, clinically and histologically, from that recorded for strain S 13, or for *Str. dysgalactiae* strain 419 (Holman, Smith and Pattison, 1953; Pattison and Smith, 1953). The main difference between them was the degree of injury to the udder tissue as shown by the fall in milk yield or by the histological changes produced. It is interesting to see that these changes were much more pronounced with the types strains, O 90 and H 36 B, obtained from human sources, than in the type strain M216 obtained from a cow and for which the udder was presumably the predilection seat.

When our strain (S 13) is compared with Lancefield's type strains, it appears to be related to the organisms obtained from human sources. In a previous paper (Pattison, Taylor and Holman, 1950) it was stated categorically that this strain was isolated in 1943 from a case of bovine mastitis. It has now been typed by Lancefield as a Ib. The similarity in pathogenicity between this strain and those of Lancefield's types Ia and Ib from human sources, and the statement by Ghoroury (1950) that type Ib had been recorded as a cause of bovine mastitits in no part of the world other than America, led us to investigate the origin of S13 which we had accepted in good faith as of bovine origin. As a result of this enquiry we are no longer prepared to assert that it is of bovine origin, nor can we say definitely that it is a human strain.

SUMMARY

Of Lancefield's four type strains of group-B streptococci, three (Ia, Ib, and II) were sufficiently lethal for mice to be used in immunity tests.

Active and passive immunity tests in mice showed that immunity was, at least in part, type-specific.

In addition to type-specific immunity, the use of the intraperitoneal route for inoculating serum or vaccine into mice provided some non-specific immunity against subsequent intraperitoneal test doses.

The histological lesions of mastitis produced by intramammary inoculation of goats with the four different type-strains of group-B streptococci were indistinguishable from those produced by strain S 13 (as previously reported) or by *Str. dysgalactiae*, strain 419.

After being given a series of subcutaneous or intraperitoneal injections of a formolised whole culture vaccine consisting of a mixture of each of the four type strains of group-B streptococci, goats were challenged by intramammary injections of one of these types. Measurable protection of the udder was demonstrated for each of the four types.

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THE BLOOD LEUCOCYTES IN INFECTIOUS MALIGNANT CATARRH OF THE OX AND RABBIT

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INTRODUCTION

During the course of studies at this laboratory on experimental infection of both cattle and rabbits with the 'D' strain of infectious malignant catarrh virus (Piercy, 1952 a, b; 1953a), advantage was taken of the opportunity to investigate the pathology and some aspects of the haematology of this disease. This paper deals with the latter. Studies of the histopathology in both species (Plowright, 1953) showed tissue changes pointing to a primary effect of the virus on the lymphoid cells and their precursors. It was to be expected that these lesions would be accompanied by changes of the blood leucocyte picture.

Reports in the literature of haematological examinations are scanty and very incomplete. Thus Marshal, Munce, Barnes and Boerner (1920) recorded that blood examinations were carried out on four fatal cases during the middle to late course of the disease. An average total of 10,000 cells per c.mm. was obtained, with differential counts of approximately 40 per cent lymphocytes, 13·5 per cent "mononuclears", 42 per cent neutrophils and 3·5 per cent eosinophils. Mettam (1923), working with South African "snotsiekte", which has since been shown to be identical with infectious malignant catarrh, recorded an increase of neutrophils, from the normal figure of 40 per cent up to 80 per cent at death with a definite "shift to the left". Eosinophils and basophils disappeared at the beginning of the reaction; monocytes showed no change. This worker concluded that the blood picture was that of an acute bacterial infection. Götze (1930) in the peracute and intestinal forms of the disease, found a "monocytosis", disappearance of eosinophils and a neutrophil shift to the left. In the "head-and-eye" form he described the same "monocytosis" and neutrophil "shift to the left" but stated that eosinophils persisted whilst a decrease in their numbers pointed to a poor prognosis.

Most of these accounts are thus mutually contradictory; none of them agrees with the findings recorded here, except in some minor points.

MATERIALS AND METHODS

Cattle

Repeated observations on the blood leucocyte picture were made in eleven animals from the 7th(4), 8th(1), 9th(2), 20th(2) and 23rd(2) laboratory passages (Table I). All these cattle were infected by

intraglandular (prescapular) inoculations of 2 c.c lymph gland suspension. In addition, single examinations on a few other animals during the period of temperature reaction (4th to 6th days) supported the findings made by daily observations.

TABLE 1.

HISTORY OF PASSAGES IN CATTLE AND RABBITS WITH THE "D" STRAIN OF INFECTIOUS MALIGNANT CATARRH.

Number	*Ox or rabbit passage*	*Inoculum*	*Route*	*Incubation period days P.i.*	*Death or destruction days P.i.*
Ox 1053	7	2·0 cc. l/gl	i/gl	18	22
,, 1012	7	,,	,,	17	21
,, 1055	7	,,	,,	17	19
,, 1048	7	,,	,,	16	23
,, 1190	9	,,	,,	19	23
,, 1016	9	,,	,,	18	22
,, 1228	9	,,	,,	23	27
,, 2356	20	,,	,,	16	21
,, 2343	20	,,	,,	16	21
,, 2613	23	3·0 cc. l/gl	,,	16	25
,, 2669	23	2·0 cc. l/gl	,,	17	56
Rabbit 84	2	2·0 cc. spleen	i/p		
		1·0 cc. brain	i/c	51	55
,, 91	3	2·0 cc. spleen	i/p	11	17
,, 92	3	,, ,,	,,	11	17
,, 89	4	,, ,,	,,	14	16
,, 90	4	,, ,,	,,	16	19
,, 93	5	,, ,,	,,	11	13
,, 94	5	,, ,,	,,	15	18
,, 95	6	,, ,,	,,	14	18
,, 98	6	,, ,,	,,	14	16
,, 19A	7	,, l/gl	,,	12	15
,, 20A	7	,, ,,	,,	11	15
,, 21A	8	,, ,,	,,	15	18
,, 22A	8	,, ,,	,,	18	21
,, 111	10	,, ,,	,,	18	21

Total leucocyte counts were carried out on jugular blood, drawn before 10 a.m., into tubes containing a mixture of dry potassium and ammonium oxalate (Whitby and Britton, 1950). Differential counts were made on smears stained by Giemsa or May-Grünwald-Giemsa methods. The latter seems to offer some advantages, especially in the definition of the granules of the neutrophils. In each film 200 cells were usually counted, in zig-zag fashion. Earlier studies showed no clear change in the monocytes, and because of the difficulty of avoiding subjective errors in the recognition of this cell type, all cells of the non-granular series were grouped together. Especially was this justifiable in view of the occurrence of abnormal cell types which appeared frequently in the course of the disease and could not easily be classified.

In this connection a quotation from Bloom (1948) may be of interest. He states, "The monocyte has not been treated with sufficient detail in our material. This is a reflection of the fact that

there is little general agreement on what a monocyte is. It would take some years of study of the material for all the individuals in our group to determine with a fair degree of accuracy which cells in the several species are monocytes and which are not, even following any particular author's criteria."

In six animals of the 7th and 9th passages, in addition to the usual daily clinical examination, the prescapular and prefemoral glands were palpated to detect the presence and degree of enlargement. This was done in an attempt to correlate changes in the blood with the clinical manifestations of the disease.

Rabbits

Daily observations were made on fourteen rabbits, some details of which are shown in Table I. Blood was withdrawn by pipette directly from the cleansed skin of the ear after a vessel had been incised with a razor blade. Total counts were made immediately. Smears were prepared at the same time and stained by May-Grünwald-Giemsa; monocytes were counted separately. In eight instances at least one bleeding was carried out prior to inoculation; in two animals (21A and 22A) it was commenced eleven days, in another two (19 and 20A) four days before inoculation. All bleedings were carried out between eight and ten o'clock in the morning. Four other rabbits were examined on one or more occasions during the period of reaction; the results were comparable with those recorded here.

It should be mentioned that *Salmonella (typhimurium)* and coccidial infections exist in Kabete rabbit stocks. Some deaths have occurred in experimental animals due to these causes alone, but latterly, in an attempt to suppress them, animals before and during experiment have been given sulphamezathine continuously (Rabbits 19 to 22A inclusive). The only rabbits for which data are recorded here are those which developed a typical temperature reaction after the usual incubation period and which, on post-mortem examination, showed characteristic lesions of malignant catarrh.

RESULTS

Cattle

Total Leucocyte Counts

No consistent or significant changes were found during the incubation period. About 24 hours after the initial temperature reaction, sometimes practically coincident with it, a depression of the total leucocyte count commenced and continued for the next 3 to 4 days (see Figs. I to III). In earlier passages the animal was by this time usually moribund or dead. Later, as exemplified by animal 2669, there appeared to be a more irregular response, an initial fall being succeeded by irregular increases, largely accounted for by polymorphonuclear leucocytosis. This animal survived for a considerably longer period.

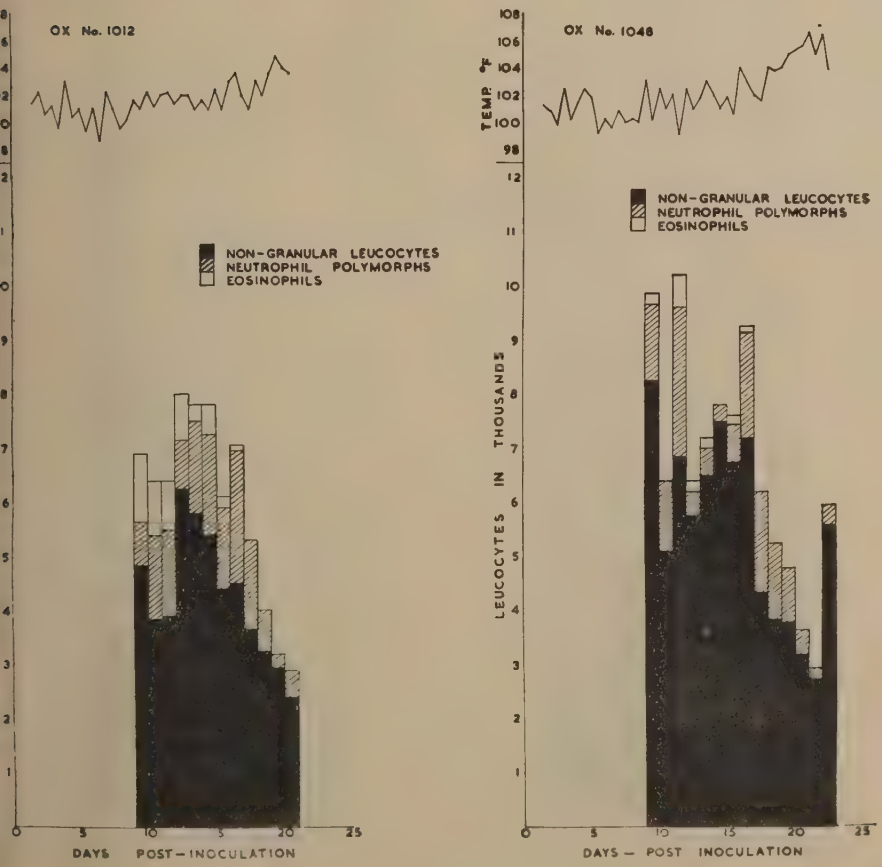
Enlargement of the gland of inoculation was usually obvious at least 1 to 2 days prior to the rise of temperature and also to generalised lymphatic gland involvement. The latter process was detectable at about the time of the first temperature elevation or within 24 hours of its commencement. Hence it may be said that fever, generalised lymphatic gland enlargement and fall of the total blood leucocytes began at about the same time. Also noticeable during this period were palpable enlargement of the haemolymph glands and a watery ocular discharge, followed one or two days later by a nasal discharge, of a serous or muco-serous type.

Differential Counts

Non-granular series

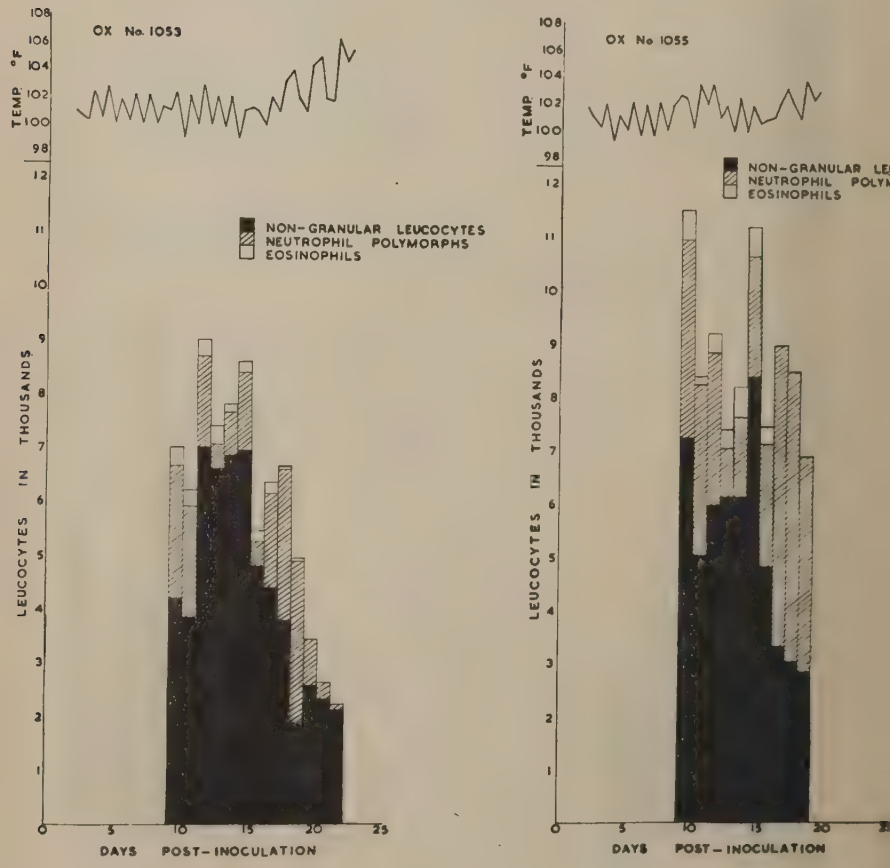
Reference to the histograms (Figs. I to III) will show that the fall

Fig. 1



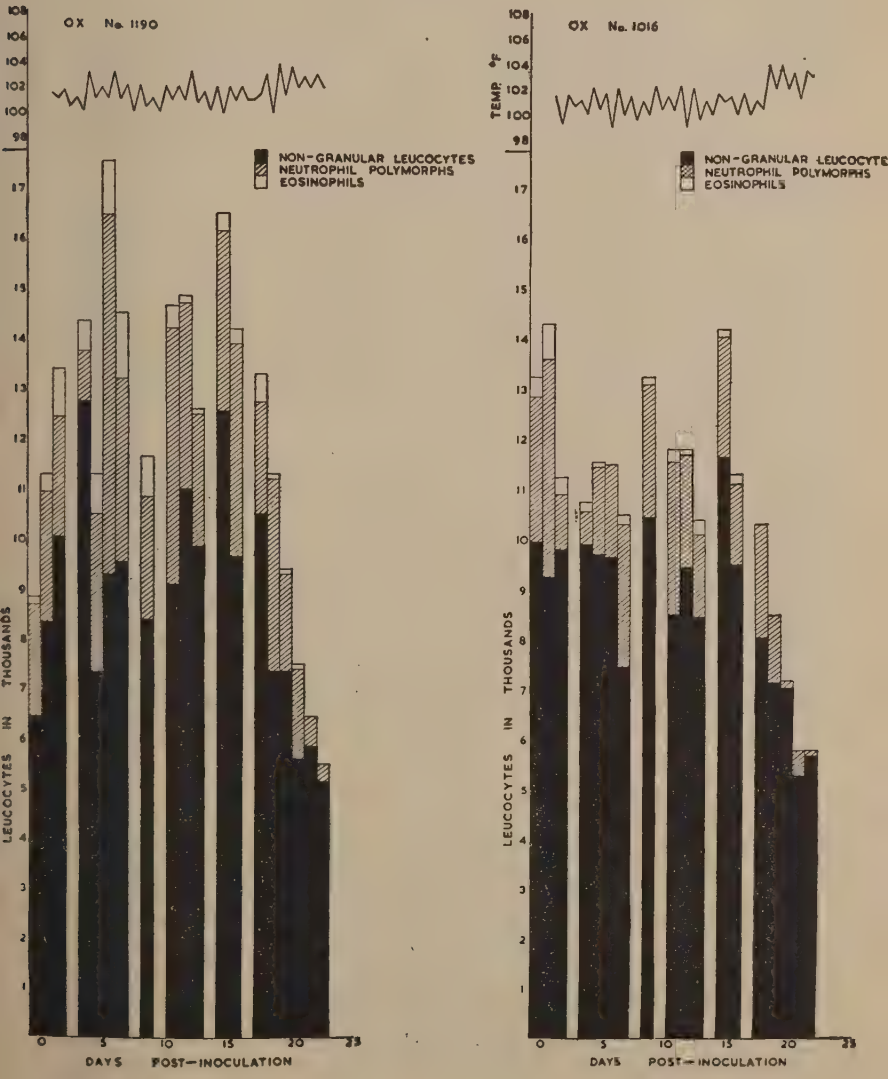
in total leucocyte counts was accompanied by a simultaneous decrease in the number of mononuclear cells which reached low figures of approximately five to two thousand per c. mm.; sometimes, as in ox 1053, the numbers dropped below 2,000. During the leucopenic phase the proportion of small lymphocytes decreased and those present were sometimes shrunken and pyknotic. There was a corresponding relative increase in the larger lymphocytes and up to 2 to 2·5 per cent of the total non-granulars were very large cells with a rounded or indented nucleus containing much coarsely granular chromatin material and with large amounts of intensely basophilic cytoplasm, somewhat irregular in outline. In many respects these cells resembled those found in large numbers in the lymph glands and elsewhere at this stage of the disease. In sections, however, the

Fig. 2



indentation of the nucleus by deep infolding of the nuclear membrane appeared to be more prominent. For want of a better name they could be called "primitive cells" and it is considered that they represent various abnormal developmental stages between reticulum cells and lymphoblasts (large lymphocytes).

Fig. 3



Neutrophil polymorphs

In three of the four 7th passage animals (1053, 1055, 1012) there was a definite increase of neutrophils above previously existing levels during the first two or three days of the reaction. A rapid decline then ensued causing a marked fall in the differential count—in 1012 to 8 per cent on day 20 post-inoculation, and 5 per cent in 1053 on day 22. In animals 1016 and 1190 differential counts on the final day showed 2 per cent and 6 per cent respectively of neutrophils, the rest being non-granulars. In later passages no such definite absolute increase of polymorphs was recorded during the period of primary reaction. It is probable that, in natural cases of the severe "head-and-eye" form of the disease, this represents the usual state of affairs —i.e., a leucopenia affecting all elements, with a marked relative increase in non-granular cells.

Whilst no attempt was made in counting to subdivide the polymorphs according to age it was quite clear that the cells present during the period of advancing neutropenia included a greatly increased proportion of bandforms and even some myelocytes, thus pointing to either increased destruction of polymorphs and/or interference with the capacity of the bone marrow to replace those which degenerate. In the spleen of cattle with malignant catarrh there is some evidence of increased destruction of neutrophils; no examinations of bone marrow were made.

In the animal 2669 (23rd passage) which was not killed until 56 days following inoculation or 40 days following the first reaction, there were periods of intermittent pyrexia with no marked change in the figures for non-granular cells, whilst the peaks of the temperature curve coincided with the neutrophil leucocytosis (Fig. IV). Post-mortem examination did not indicate the presence of any intercurrent infection to account for this. A similar remittent fever has been seen more recently in a 33rd passage animal which was allowed to survive until day 59; it also showed only lesions of malignant catarrh at post-mortem examination, whilst virus was present in the blood on the 51st day and in the lymph glands at slaughter (Piercy, 1953b).

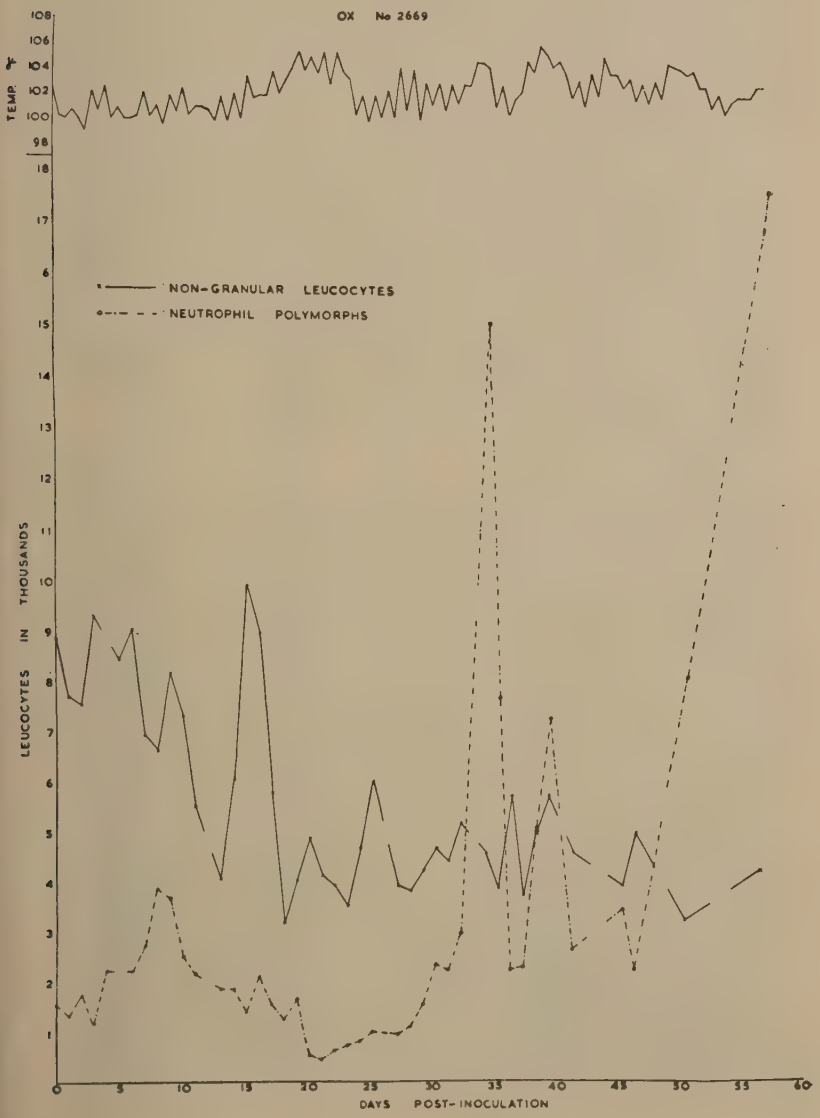
Eosinophils

During the period of primary reaction there was a sudden reduction in the number of eosinophils. In all animals except those of the 23rd passage they disappeared completely in 8 of 9 instances within 24 hours of the first rise of temperature, and after 48 hours in the remaining animal (1190). In the two 23rd passage cattle an initial depression was followed by a partial recovery, but the total number of eosinophils did not regain the pre-reaction levels and in 2669 the periods of neutrophilic leucocytosis were accompanied by their complete disappearance.

Basophils

These were present in three animals but showed no significant changes in relation to the disease.

Fig. 4



Rabbits

Total Leucocyte Counts

The immediate effect of the intraperitoneal inoculations was usually to produce a mild polymorphonuclear leucocytosis which subsided rapidly. In fact, the total count 48 hours after inoculation was often below the pre-inoculation figure. No consistent changes occurred during the incubation period but, on the appearance of the temperature reaction, two distinct types of leucocytic response were found to ensue.

In the first, Type I (Figs. V and VI), which was observed in nine of the 14 rabbits (with one exception, No. 92), leucocytosis developed with maximum total counts in the range 15 to 30,000 cells per c. mm. or even more (35,500 in rabbit 95). In two of these nine (91 and 92) the leucocytosis was preceded by a definite depression, falling to 7200 and 3450 respectively, the former being in fact a terminal phenomenon. (Fig. VI.) In the second kind of response (Type II) as seen in 111, 20A and 22A, the total count fell progressively from the beginning of the reaction with final figures of 5550, 4050 and 5600 per c. mm. respectively (Fig. VII). In the two remaining rabbits (84 and 89) the total counts did not change appreciably, but 99 was killed on the 2nd day only of the disease.

Differential Counts

Lymphocytes

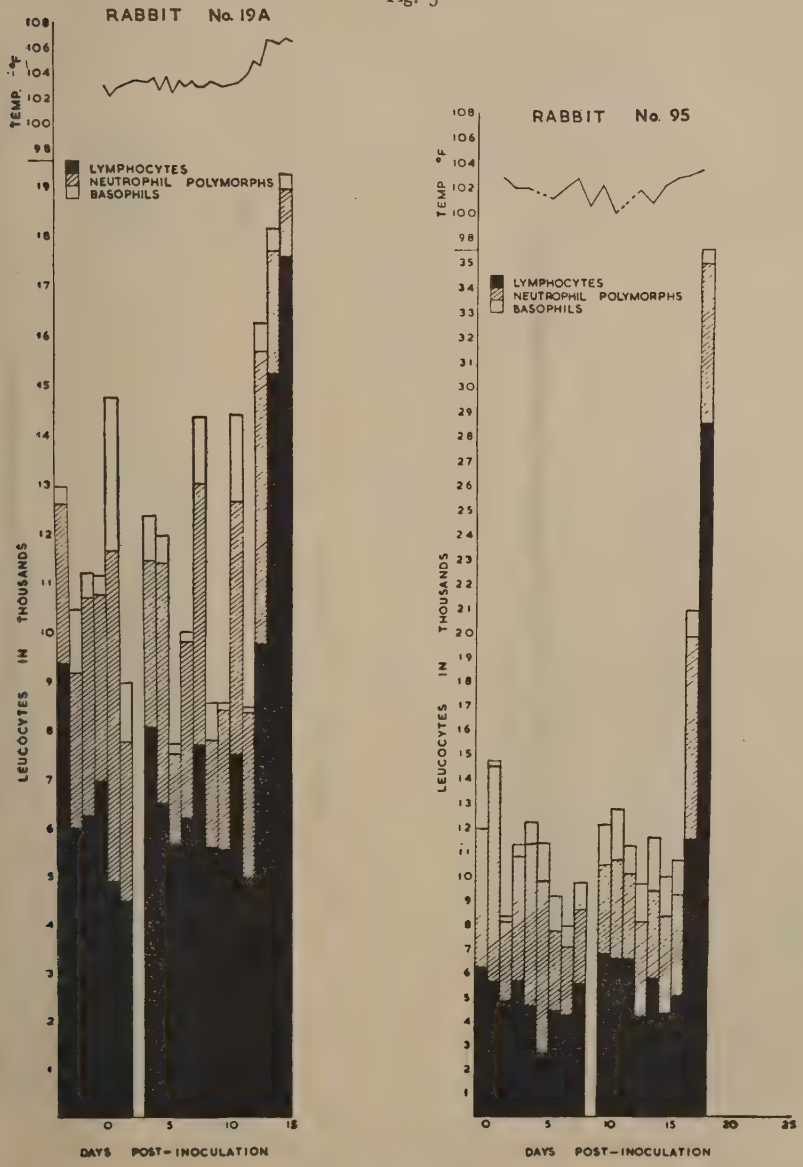
As noted already, intraperitoneal inoculation is often succeeded by a leucocytosis, which is usually accompanied by a slight depression or no change in the number of lymphocytes and hence a decline in their percentage, which lasts about 24 to 48 hours.

With the appearance of the temperature reaction, or immediately preceding it in Type I, there may be a transitory slight decrease in lymphocytes, as in 93, 19A and 21A. In 91 and 92 figures for lymphocytes remained low during the first six days of the disease and then increased very rapidly, to 15,600 and 11,750 per c. mm. respectively. In other animals a lymphocytosis of 10,000 cells or more per c. mm. was attained after 24 hours (3), 2 days (2) or 3 days (2). Four animals exceeded 15,000 and two, 20,000 lymphoid cells per c. mm. In leucocytic reactions of Type II a moderate to mild decrease of lymphocytes persisted throughout.

In the nine animals of Type I all but one showed over 80 per cent. lymphoid cells on one or more occasions during the period of lymphocytosis; in four instances a maximum of over 90 per cent was recorded. Of the three rabbits of Type II, two (111 and 20A) showed a proportion of lymphocytes increasing with the depression in their total number to a maximum of 75.5 per cent and 85 per cent respectively. The third, (22A) showed an initial decrease to 33 per cent on the penultimate day, with rapid change to 72 per cent on the day of destruction. In 89, the proportion was increasing; there was no definite trend in 84.

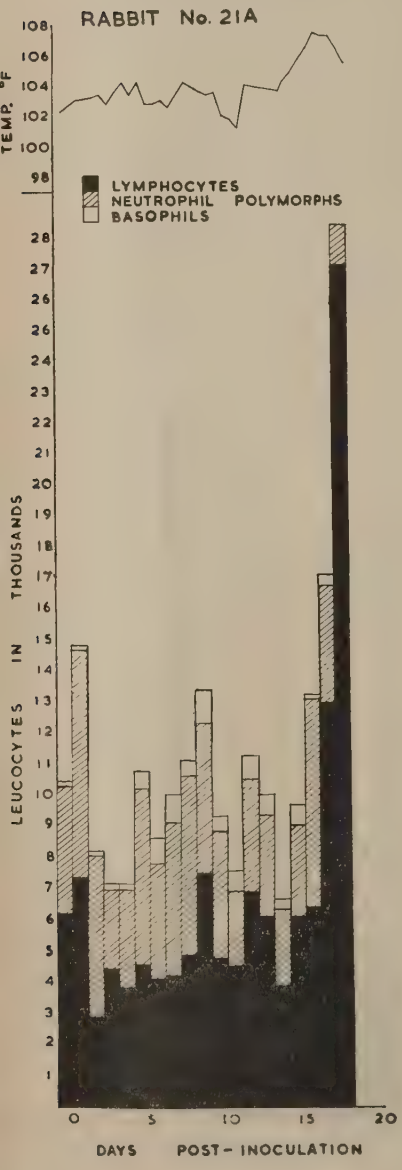
Changes in the numbers of lymphoid cells were accompanied by alterations in their morphology. In all animals, from the time of the temperature reaction, there were increasing numbers of cells which

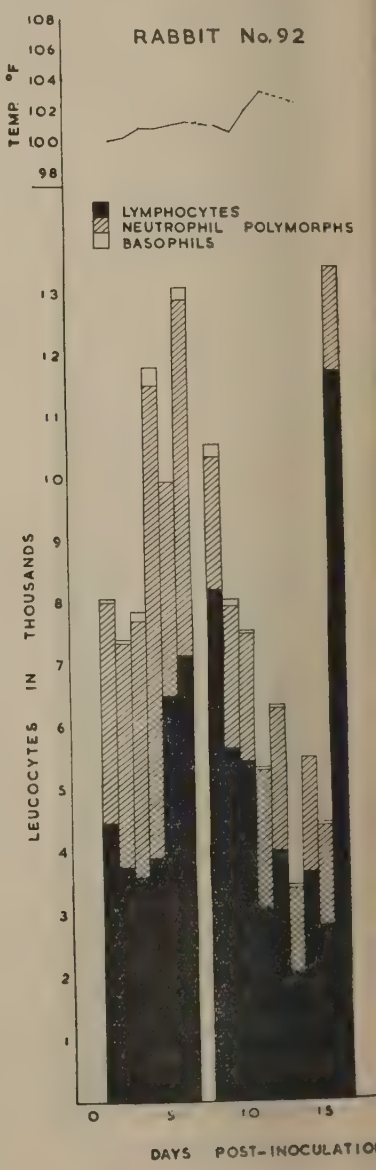
Fig. 5



appeared to be very similar to those described as Type I by Downey and McKinlay (1923) in infectious mononucleosis of man. In smears

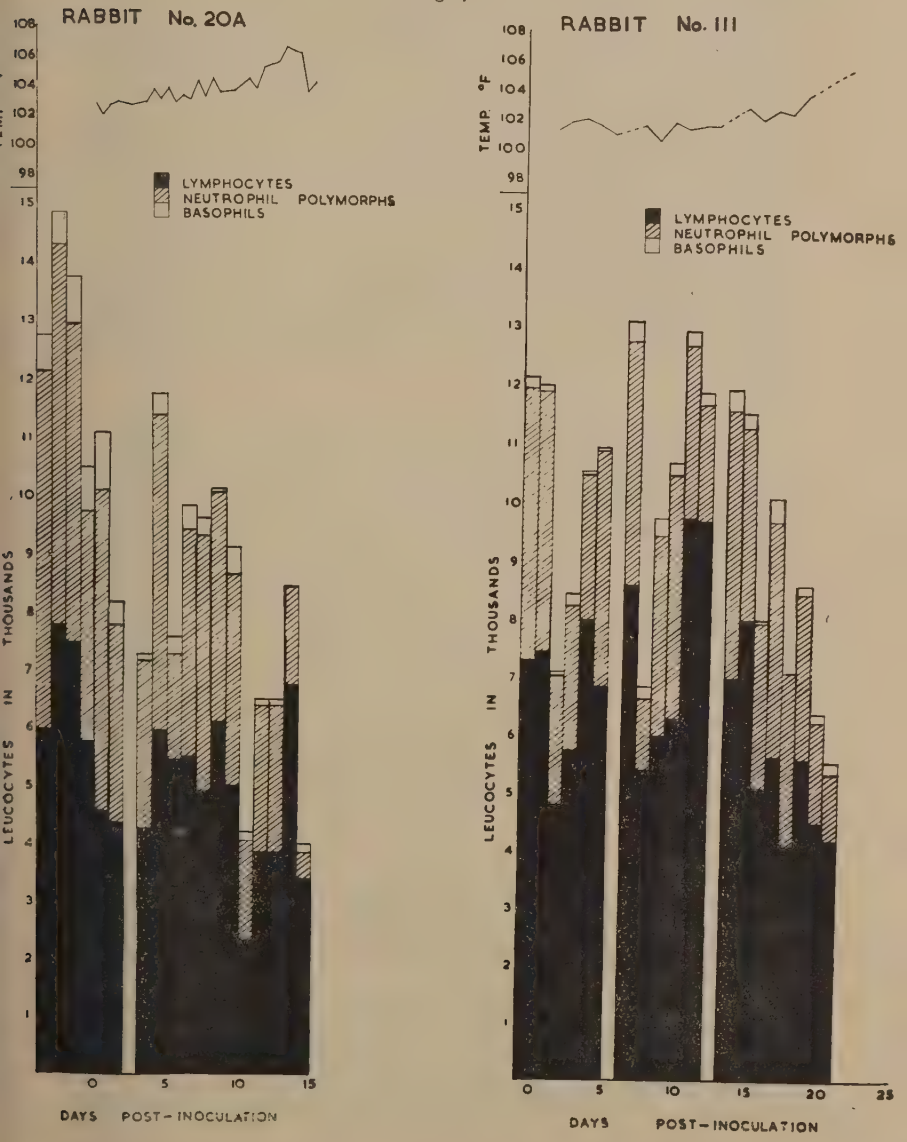
Fig. 6





stained by Giemsa or May-Grünwald-Giemsa these were about the size of the normal medium or large lymphocyte or even larger. The nucleus was often flattened or slightly indented and displaced to one

Fig. 7



side of the cell; very little of its structure was visible. There were no distinct nucleoli and no clear separation of chromatin strands and masses, giving a dark, cloudy appearance. The cytoplasm, especially in the larger cells, often showed rounded projections resembling pseudopodia, and in these and along the cell membrane generally there was a condensation of material staining deep china-blue or more frequently, a slatey-blue colour. The cytoplasm surrounding the nucleus was, on the contrary, often almost clear and pale. Azurophil granules were commonly scattered in the cytoplasm, especially in those with less marked perinuclear clearing and many cells showed small rounded cytoplasmic vacuoles. Occasionally, nuclear fenestrations, as described by Osgood (1935), were observed and mitotic figures could also be found.

In several animals, but especially in 19A and 20 A, it was evident that many of the circulating small lymphocytes at the beginning of the reaction were shrunken and degenerating. They were no bigger then erythrocytes and it was difficult to distinguish the extremely narrow cytoplasmic ring which surrounded the very dark pyknotic nucleus. Superficially, these cells resembled the extruded nuclei of normoblasts.

In addition to these abnormal lymphocytes, cells, which were undoubtedly macrophages, appeared in some cases in the peripheral blood. They were very large elements with a ragged outline and abundant pale-staining cytoplasm which included much cellular debris; the nucleus had the usual characteristics. These cells are identical with those which appear in enormous numbers in the lymph glands during the phase of destruction of small lymphocytes.

As to the proportion of the "large mononuclears", they were counted on many occasions as a percentage of the total non-granulars. They appeared at about the time of the temperature reaction and increased rapidly to constitute 40 to 90 per cent approximately of this group, but there was no apparent direct relationship between the total and the percentage of large mononuclears. Macrophages did not usually exceed 1 to 2 per cent of the non-granular "leucocytes" although in rabbit 91 they formed about 5 per cent.

Neutrophils

The polymorphonuclear leucocytosis which often followed inoculation lasted for about 24 hours and then there was a tendency for the neutrophils to fall temporarily below preinoculation figures (20A, 22A).

On the day preceding the temperature reaction there was a moderate and transitory absolute increase in polymorphs in three animals (89, 98, 22A). In two of these (98 and 22A) the figures were depressed on the first day of reaction but reached levels high for the individual on the second and third days respectively; in 98 there was

a persistence of the polymorph on the first and second days. A further four animals showed definite high levels for polymorphs on the 2nd (94, 19A, 21A) and 4th days of the disease, so that altogether an absolute increase in polymorphs was clearly demonstrated just before and/or during the reaction in 7 of 14 rabbits. At the time of death or destruction 9 animals showed a neutropenia, the figure including all three individuals with a Type II (leucopenic) response. No rabbit showed a terminal polymorphonuclear leucocytosis.

Again no attempt was made to subdivide the neutrophils whilst counting but it was obvious that an increasing proportion of them during the reaction were young forms and especially was this the case during the final neutropenia.

Monocytes

The only important change which these cells showed was an increase in number 24 to 48 hours after intraperitoneal inoculation and a tendency to disappear completely later in the course of the disease reaction. This applied equally to animals with terminal leucopenia or lymphocytosis.

Basophils

Throughout observations on the blood leucocyte picture of rabbits at Kabete, with malignant catarrh as also with lapinised rinderpest (Plowright, 1952), there has been a remarkable fluctuation in the number of basophils both from day to day in the same individual and between different animals. There have also been rabbits which have shown figures for basophils which were consistently higher than the normally accepted range, e.g. 95 showed 10 per cent or more from the fifth to the sixteenth day following inoculation. Prior to the time of the reaction there has usually been no obvious relationship between the numbers of basophils and those of any other cell type.

In five of the fourteen animals a reduction of basophils took place during the course of the disease to the extent that none was present at the time of destruction. In a further three animals no basophils were present on the 3rd day of the reaction and in one they disappeared temporarily on the 1st day; in all four of these rabbits basophils had been consistently present during the incubation period. Hence, a clear-cut depression of basophils was observed in 9 out of 14 animals.

Eosinophils

Since these cells were not consistently present in any animal and rarely exceeded 1·5 per cent of the total, the significance of changes in their numbers is more difficult to assess. However, in only two of the fourteen rabbits were the eosinophils present at the time of death or destruction. These cells are not included in the histograms.

DISCUSSION

In view of the prominent lesions which occurred invariably in the lymphopoietic organs of both cattle and rabbits with malignant catarrh, it appeared probable that the blood leucocytes would show marked and simultaneous changes. In cattle, the surprising feature was that considerable proliferative activity of lymphoid cells and their precursors was not accompanied by an absolute mononucleosis. It has been shown, at least up to the 23rd artificial passage, that there is always during the acute phase a leucopenia which affects both granular and non-granular elements; in one animal which survived 40 days after onset of the disease the lymphoid cells never returned to their previous level but there was a remittent fever characterised by polymorphonuclear leucocytosis.

In rabbits on the contrary, whilst the behaviour of individual animals varied considerably, a large proportion showed a terminal mononucleosis with flooding of the circulation by cells similar to those which appeared in enormous numbers in the lymphopoietic organs and elsewhere. These cells appeared to be atypical, but not immature, large lymphocytes. Their escape into the blood vascular system was probably aided by the frequent lesions of blood-vessel walls, especially of venules, these lesions often extending to and involving the intima (Plowright, 1953). In cattle, similar lesions of blood vessels were present less frequently but very large numbers of large basophilic cells of large lymphocyte type were often observed within the blood vessels and sinusoids of the lymphopoietic and other organs. Some unknown factor appeared to prevent their liberation into the lymph and bloodstreams in numbers sufficient to overcome the fall in non-granular leucocytes caused by the early destruction of smaller lymphoid cells.

It is remarkable that malignant catarrh and acute East Coast fever, respectively viral and protozoal diseases of the ox, each characterised, at one stage at least, by marked hyperplasia of lymphoid tissue and proliferation of lymphoid cells elsewhere, are both accompanied by a progressive leucopenia (Steck, 1928; Barnett, 1953). In acute cases of East Coast fever the leucopenia also progresses to death, at which time the total leucocyte counts are commonly down to 1,000 cells per c. mm. or even less—somewhat lower than those encountered in malignant catarrh. Barnett (1953) also finds that in acute *Theileria parva* infections occasional animals may show a secondary lymphocytosis before death; this is reminiscent of the reaction in some rabbits. In both diseases leucopenia affects all cell types, the proportion of neutrophils showing a marked decline towards the termination, those which are present being increasingly young forms.

The behaviour of the leucocytes in the rabbit shows many similarities to that observed in man during attacks of infectious mononucleosis (Whitby and Britton, 1950; Kracke, 1941) except, of

course, that in the rabbit the infection is an acute, highly fatal one. There are, for example, the variability in response, the cases of initial leucopenia, the large proportion of individuals with a relative and absolute increase in the number of "mononuclear" cells during the period of lymph gland enlargement and the initial polymorphonuclear leucocytosis in some instances. In addition there are the morphological resemblances already noted between the abnormal cells in both conditions.

The study of blood changes in cattle and rabbits gives strong support to the hypothesis that the virus of malignant catarrh has an initial destructive effect on the smaller lymphoid cells. In many rabbits the secondary stimulation of mesenchymal reserves both in the lymphopoietic organs and elsewhere is reflected in the liberation of large numbers of abnormal large lymphocytes into the circulation.

SUMMARY

The results are recorded of repeated examinations of the blood leucocyte picture in eleven cattle and fourteen rabbits infected with a virulent "head-and-eye" strain of bovine infectious malignant catarrh.

In cattle there was a progressive leucopenia commencing at about the same time as generalised lymphatic gland enlargement. The leucopenia affected all cell types, but there was a relative mononucleosis, with the appearance of large primitive cell-types. In one animal which survived 40 days following the first reaction there was recurrent fever with polymorphonuclear leucocytosis.

In rabbits the response varied. In nine there was a leucocytosis, in three a mild leucopenic response, in two no significant change in total counts. The leucocytosis was due to large numbers of cells which appeared to be abnormal large lymphocytes. In all cases there was an increasing proportion of non-granular cells during the reaction.

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RUBARTH'S DISEASE
(Canine Virus Hepatitis)
II. The Insusceptibility of Ferrets to Experimental Infection

By

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INTRODUCTION

For several years now most workers have regarded the ferret as a very useful animal in drawing a distinction between the viruses of the canine distemper-complex (DCX) and the virus of Rubarth disease, canine hepatitis (H.C.C.). Green (1940) was the first to draw attention to this difference and amongst a number of other workers who have failed to infect ferrets with H.C.C. are Parry and Larin (1951).

In 1950 Goret, Joubert and Buffet reported that they had succeeded in infecting ferrets and in passaging H.C.C. virus in this species. Later, in 1951, Goret, Lucam, Joubert, Flachat and Ott, claimed to have reproduced the disease in dogs with material of the 6th and 22nd ferret-passages.

For any worker studying the inter-relationship of the virus diseases of dogs, the susceptibility of the ferret to H.C.C. is clearly a matter of great importance and we have, therefore, thought the subject worthy of detailed investigation. We have received much assistance from Professor Goret and we wish to express our thanks to him for his co-operation in supplying strains of virus and in bringing us some ferrets of French origin.

MATERIALS AND METHODS

The materials used as sources of virus have been of dog and of ferret origin. The dog material has, with the exception of the "Beckenham" strain received from Mr. Parry of the Animal Health Trust, Canine Research Station, consisted of small pieces of dog liver in glycerine sent by Professor Goret. On different occasions three strains, the "Fromme" fox encephalitis, the "Rubarth" and the "Florent-Leunen" have been received by post. The "Beckenham" strain consisted of a specimen of freeze-dried liver. The ferret-passaged virus from Professor Goret has consisted of the liver and spleen of ferrets of the 2nd passage of "Fromme", the 3rd passage of "Rubarth", 6th "Rubarth", 13th "Fromme" and 22nd "Florent-Leunen". Some of these tissues have been received in glycerine, others freeze-dried.

We are fortunate in being able to isolate our ferrets in accommodation on three different sets of premises. One farm is used as a

TABLE I

SUMMARY OF RESULTS OF INOCULATING DOG MATERIAL TO FERRETS

Experiment Number	*Date*	*Strain and Source*	*Result of C.F.T. on Original Material*	*Result of Ferret Inoculation*	*Result of Challenge*	*Result of C.F.T. on Ferret-Tissue*	*DCX*	*H.C.C.*
1	21.6.51	Liver in glycerine Goret		Four English ferrets 246-249 i/p. No thermal or clinical reaction.	Challenged 54th day with ferret passaged virus X F. 250-1. Reacted and died with distemper symptoms.	See experiment 1(a) Table II		
2	24.9.51	"Fromme" 4th Liver in glycerine Goret		French ferrets 281, 282 and English ferret 283 i/p. No reaction. F.282 and 283, inoculation repeated. No reaction.	F.281 and 282 challenged 65th day with distemper. Reacted. F.283 challenged with Goret's "ferret adapted H.C.C.". Reacted and died with distemper symptoms.	F.281 F.282 F.283	+ + +	+ tr. —
3	24.9.51	"Florent-Leunen" 6th Liver in glycerine Goret	+H.C.C.	French ferrets 284-5 and English ferret 286 i/p. No reaction. F.285-286, inoculation repeated. No reaction.	F.284 and 286 challenged 65th day with distemper, reacted. F.285 challenged with Goret's "ferret-adapted H.C.C.". Reacted and killed *in extremis* with distemper symptoms.	F.284 F.285 F.286	+ + +	+ tr. +
4	24.9.51	"Rubarth" 4th Liver in glycerine Goret		French ferrets 287-8 and English ferret 289 i/p. No reaction. F.288 inoculation repeated. No reaction. F289 killed accidentally when bleeding.	F.287 and 288 challenged 65th day with Goret's "ferret—adapted H.C.C." Reacted and killed with distemper symptoms.	F.287 F.288 F.289	+ + +	tr. tr. —
5	24.9.51	"Beckenham' Liver freeze-dried Parry and Larin	+H.C.C.	French ferrets 290 and 291 and English F.292 i/p. No reaction. F.291 inoculation repeated. No reaction. F.292 killed when bleeding.	F.290 and F.291 challenged 65th day with Goret's "ferret adapted H.C.C." Both reacted and killed with distemper symptoms.	F.290 F.291 F.292	+ + —	+ — —

6	18.2.52	"Rubarth" 7 Liver in glycerine Goret	+H.C.C.	English ferrets 232 and 344 s/c. F.345 and 346 i/c and i/p and F.347 and 348 i/p. No reaction. F.346 died 10th day and F.347 on 42nd day from causes not connected with the inoculation.	F.343 challenged 35th day "Green" distemper-strain, reacted and killed 7th day. F.344 challenged with "Glasgow" reacted and killed 9th day. F.345 died 12th day and 348 killed *in extremis* 15th day with distemper symptoms.	F.343 F.344	+ +	— —
7	25.2.52	"Fromme" 7 Liver in glycerine Goret	+H.C.C.	English ferrets 354 and 355 i/c and i/p. 356 and 357 a/c and 358 and 359 i/p. No reaction.	Used for distemper investigation after 35 days.			
8	24.3.52	"Fromme" 7 Liver in glycerine Goret	+H.C.C.	French ferrets 371, 373 i/p, 372 a/c and English ferret 374 i/p. No reaction. All ferrets received a second dose on the 7th day. No reaction.	Used for distemper investigation after 24 days, proved to be susceptible to the distemper complex.			
9	24.3.52	"Florent-Leunen" Liver in glycerine Goret	+H.C.C.	French ferrets 375 and 376 and English ferret 377 i/p. F.375 killed 7th day in an attempt to increase the virulence of the virus by passing an emulsion of its spleen and liver into French ferrets 386 and 387 i/p. No reaction. Ferrets 376 and 377 received a second dose on the 7th day. No reaction.				
10	24.3.52	"Rubarth" 7 Liver in glycerine Goret		French ferrets 378 and 379 and English ferret 380 i/p. All three received a second dose on 7th day. No reaction.				

clean centre, that is to say on it are housed ferrets that have been inoculated, but are not expected to react to a canine distemper-complex (DCX) virus. The temperatures are taken daily and immediately a ferret shows a thermal reaction or any symptom suspicious of distemper it is removed at once to the infected centre. This is another farm on which four isolation stables are available. Ferrets inoculated with a preparation likely to produce a reaction to distemper are placed in one of these stables and we have found over a period of six years that the isolation between the different stables on this farm has proved quite remarkably efficient. Stock, uninoculated ferrets are kept at a third centre, the laboratory.

Complement-fixation tests have been used to determine the types of antigen present in various materials and on one occasion to examine sera for antibodies. The method adopted was that to be described by Mansi. There is no cross reaction between H.C.C. and DCX.

RESULTS

Inoculation of Virus (H.C.C.) from Dogs to Ferrets

In all, some 10 attempts have been made to infect ferrets with H.C.C. using infective material of dog origin. Four different strains of virus have been used; but in none of the 38 ferrets inoculated have we produced a thermal reaction or other clinical sign of infection. Fourteen French ferrets kindly given to us by Professor Goret are included in this total. Details of the inoculations will be found in Table 1 from which it will be seen that a variety of methods of inoculation were tried and that some ferrets, at the suggestion of Professor Goret, received repeat inoculations.

We have also tried to establish the virus in the ferret by killing two ferrets seven days after inoculation and inoculating a suspension of liver and spleen to further ferrets (386 and 387). No reaction took place. Both were inoculated a month later and found to be susceptible to DCX. Finally, having been fortunate enough to obtain a litter of young wild foxes we decided to see whether we could recover virus from ferrets five days after inoculation. Two ferrets (389 and 390) inoculated intra-ocularly with H.C.C. "Beck" strain developed neither thermal nor clinical reaction. The ferrets were killed on the 5th day and their brains and spleens were removed, ground with broth and inoculated intra-ocularly into a further two French ferrets (391 and 392) and to fox 3. This fox showed no local clinical reaction in the inoculated eye. Ferrets 391 and 392 were killed on the 5th day and their brains and livers were ground with broth and inoculated intra-ocularly into fox 4. Again there was no clinical response. Foxes 3 and 4 were subsequently challenged intra-ocularly with the original dog material. Fox 3 showed unilateral corneal opacity within a few seconds but this immediate effect disappeared within the following 24 hours. Fox 4 developed unilateral

corneal opacity after 24 hours. The opacity was very marked after 48 hours and lasted for three weeks. Otherwise the fox remained clinically normal.

The original inoculum used in the first pair of ferrets and for challenging the foxes was inoculated intra-ocularly to five dogs, three susceptible and two immune to H.C.C. The three susceptible dogs 54, 55 and 56 all developed a thermal reaction after 48 hours. Dogs 54 and 55 showed severe oedema of the conjunctiva and the *membrana nictitans* but recovered by the 7th day after inoculation. Dog 56 showed unilateral corneal opacity after 48 hours and was killed *in extremis* on the following morning. Neither of the immune dogs (37 and 46) gave a thermal reaction but 37 developed within 24 hours a unilateral corneal opacity which disappeared two days later.

It is clear from this experiment that the virus of H.C.C. did not survive a second passage in ferrets. It is possible that a small quantity of virus was present on the 5th day in the original pair of ferrets. This quantity of virus was sufficient to sensitise fox 3 but insufficient to produce a typical reaction in the cornea.

Inoculation with Ferret-passaged Virus

When we inoculated ferret-passaged material received from Professor Goret to our English or French ferrets we produced in the first four experiments, involving 15 ferrets, a condition clinically suggesting infection with a virus of the distemper-complex (Table 2). When we subinoculated from these ferrets to DCX-susceptible and DCX-immune ferrets we found in every instance that the distemper immune ferrets resisted the infection which proved fatal to the previously exposed ferrets. In the second four experiments we provoked no reaction in the 9 inoculated ferrets. We would emphasize that the ferrets that contracted the DCX-like illness in these experiments did so on our clean farm where they were housed with ferrets inoculated with virus-containing material of dog origin and with other ferrets, none of which developed any clinical illness. At this point we would also draw attention to the "Result of Challenge" column in Table 1. In this column it will be noticed that ferrets inoculated with dog material were all susceptible to DCX on challenge and that the ferrets challenged with ferret-passaged H.C.C. material had apparently gained no resistance from the previous inoculation of H.C.C. virus of dog origin.

Examination of the requisite columns of tables 1 and 2 will show that whereas the material of dog origin, as used for inoculation, fixed complement in the presence of H.C.C. antiserum and not in the presence of DCX-antiserum, the ferret-passaged material that we received always fixed-complement in the presence of DCX-antiserum, but on only one occasion in the presence of H.C.C. antiserum. It will also be noticed that examination of material from ferrets inoculated first with H.C.C. virus of dog origin and then a

TABLE II
SUMMARY OF RESULTS OF INOCULATING FERRET MATERIAL TO FERRETS

Experiment Number	*Date*	*Strain and Source*	*Result of C.E.T. on Original Material*	*Result of Ferret Inoculation*	*Result of Challenge*	*Result of C.F.T. on Ferrets Tissues*	*D.C.X.*	*H.C.C.*
1	27.6.51	"Florent-Leunen" 22nd (Goret)		English ferrets 250-251 i/p. Both ferrets reacted. F.250 killed *in extremis* 9th day, and 256 died 13th day with distemper symptoms.		250	+	+
						251	+	+
	14.8.51	"Sub" (250 and 251) Fresh	+H.C.C. +D.C.X.	English ferret 268, distemper-immune ferrets 56, 230 and ferrets 246, 247, 248 and 249 (which had previously received "Florent-Leunen" dog material and produced no reaction). F.56 and 230 produced no reaction. F.268 (susceptible control) died 13th day with distemper symptoms. F.247 and 248 which received anti-distemper ferret serum at time of inoculation died 17th and 23rd days respectively with distemper symptoms. F.246 and 249 died on the 10th day with typical distemper symptoms.		F.246	+	—
						F.247	+	—
						F.248	+	—
						F.268	+	—
						F.249	+	—
2	21.7.51	"Fromme" 2nd (Goret) Freeze-dried		English ferrets 254 and 255 and distemper immune ferrets 64 and 144 i/p. F254 died 12th day, 255 died 14th day with distemper symptoms. F.64 and 144 produced no reaction.		F.254	+	—
						F.255	+	—

3	21.7.51	"Fromme" 13th (Goret) Freeze dried		English ferrets 256, 257 and distemper immune ferrets 175 and 228 i/p. F.256 and 257 developed typical distemper and were killed *in extremis* 12th, 14th respectively with distemper symptoms. F.175 produced no reaction. F.228 died with retention of urine 3 days after inoculation.		F.256 F.257	+ +	— —
4	29.5.52	"Rubarth" 3rd (Goret)		French ferrets 395 and 396 i/p, received 2nd dose on 7th day. F.396 developed no thermal reaction but on the 16th day it showed mouth lesions purulent eye discharge with slight hardening of the pads. The animal was killed, spleen and liver were collected. F.395 showed no definite thermal reaction and on the 23rd day it showed atypical symptoms of distemper and was destroyed.		F.395 F.396	+ +	— —
	23.6.52	"Sub" F.296 Fresh		French ferret 401, English ferret 402 and English distemper-immune ferrets 384 and 385 i/p. The first two showed thermal and clinical distemper and were killed *in extremis* 16th and 15th days respectively.		F.401 F.402	+ +	Tr. Tr.
5	28.6.52	Goret in glycerine	+DCX traces H.C.C.	French ferrets 405, 406 i/p. No reaction. F.406 reinoculated i/p after 42 days with pool material. Sample No. 8. No reaction.	F.405 challenged with "Green" distemper after 30 days, developed thermal and clinical reactions and killed *in extremis* 10th day with distemper.	F.405	+	—

[*Continued overleaf*

TABLE II—*Continued*

SUMMARY OF RESULTS OF INOCULATING FERRET MATERIAL TO FERRETS

Experiment Number	*Date*	*Strain and Source*	*Result of C.E.T. on Original Material*	*Result of Ferret Inoculation*	*Result of Challenge*	*Result of C.F.T. on Ferrets Tissues*	*D.C.X.*	*H.C.C.*
6	2.7.52	Goret 4th in glycerine	+DCX —H.C.C.	French ferret 407 and English ferret 408 i/p. No reaction. P.408 re-inoculated i/p after 38 days with sample No. 8. No reaction.	F.407 challenged with "Green" distemper after 26 days, Reacted, killed *in extremis* with distemper symptoms 10th day.	F.407	+	—
7	7.7.52	"Rubarth" 7 6th in glycerine	+DCX	English ferret 411 and English distemper-immune ferret 365. No reaction.	Both ferrets challenged with "Green" virus i/p 25.8.52. F.411 developed thermal and clinical reactions and killed *in extremis* 11th day with distemper. F.365 remained clinically normal.			
8	9.8.52	Goret I-VII (pool) in glycerine	+DCX	English ferrets 426, 427 and 406, 408 (previously mentioned under experiment 5 and 6 respectively. No reaction.	F.427 challenged with "Green" virus i/p. 18.9.52, developed thermal and clinical reactions and killed *in extremis* 11th day with distemper. F.406, 408 and 426 used for distemper investigation and proved to be susceptible to the distemper complex.			

DCX virus showed that these samples gave fixation in the presence of both H.C.C. and a DCX antiserum. On the other hand material from most of our 450 ferrets that have died after inoculation with ferret-passaged DCX-virus gave no fixation in the presence of H.C.C. anti-serum although they all without exception contained DCX-antigen.

It will be observed that, in experiments 2, 3, and 4 of Table 2, the ferrets were inoculated with freeze-dried tissues sent by Professor Goret, but in experiments 5 to 8 our inoculum was prepared from material received by post in glycerine. This probably explains why in the first group of experiments the ferrets reacted whereas in the second group they did not. We would stress that dog liver in glycerine received from Professor Goret produced H.C.C. on inoculation to dogs and foxes and from reports in the literature we should expect this virus in glycerine to withstand exposure to room temperature for a few days. The viruses of distemper complex are, however, very fragile and do not survive long at room temperature unless freeze-dried.

The fact that in the last four experiments the virus sent in glycerine, although non infective to ferrets, gave positive reaction with the DCX-antiserum, in the complement-fixation test, is of interest. The ferrets were not immunised as the administration of a subinfective dose of virulent distemper virus does not stimulate any significant degree of resistance in ferrets (Laidlaw-Dunkin 1928).

DISCUSSION

The experiments detailed show that we have failed to adapt any of a number of strains of H.C.C. virus to ferrets. We have tried using ferrets of French origin since Goret and Lucam (1951) reported the susceptibility of the French ferret (*Putorius furo,* L.). We have also altered the diet from the bread and milk used as a routine in this laboratory to milk and raw meat as used by Professor Goret. At Professor Goret's suggestion we have made attempts to infect ferrets at different times of the year in case the susceptibility varied with the season. We have tried different routes of inoculation, intracerebral, intraperitoneal, subcutaneous and intra-ocular, and we have in some instances repeated the inoculation within a week. We have used small and large doses. None of these conditions has enabled us to produce clinical evidence of infection in ferrets inoculated with virus of canine origin.

Our single experiment using foxes as indicators of the presence of virus, showed that a trace of virus only was present in two ferrets 5 days after inoculation with H.C.C. virus of proved infectivity. Subinoculation to further ferrets resulted in loss of the virus completely 5 days later. This experiment hardly suggests that H.C.C. virus can multiply in the ferret. Foxes inoculated intra-ocularly using the technique described by Evans, Yanamura and Green

(1943) provide a sensitive test for the presence of H.C.C. virus in an inoculum.

Our observations on Professor Goret's ferret-passaged strains would appear to show that these strains produced a condition clinically and immunologically indistinguishable from DCX-virus infection. We are confident that our isolation facilities are sufficiently good to permit us to insist that the disease that developed in our ferrets was actually due to the inoculum and not to chance infection; in addition we have been able, by using the complement-fixation test, to demonstrate that the strains, when received by us here, were already good distemper (DCX) antigens. We are, therefore, unable to confirm the findings of Goret, *et. al.* (1950, 1951) that the disease which they passaged in ferrets is H.C.C.

In view of our findings it is difficult to understand how Goret, *et, al*, (1951) appear to have produced H.C.C. on inoculating dogs with material of the 6th to the 22nd ferret-passages. There is, however, evidence to show that this virus often produces a mild or subclinical infection in dogs and that it will persist in recovered animals for long periods (Parry, Larin and Platt, 1951). Poppensiek and Baker (1951) recovered virus from the urine of dogs for at least 161 days after infection. It is very possible that the inoculation of distemper virus to a dog harbouring a latent infection with H.C.C. virus would produce a flare-up and no doubt in such dogs, dying within a few days of inoculation, demonstration of the characteristic inclusion bodies might be possible. One also notes from the table given in the paper by Goret, *et. al.* (1951) that inclusion bodies were not found in dogs inoculated with actual ferret-material except of the first passage. With serial passages in dogs there would be the possibility that a latent H.C.C. virus was picked up and transferred to a susceptible dog. Without the evidence of a serological examination we ourselves would never feel sure of controlling our experiments. Rubarth (1947) and Parry, *et. al.* (1951) also consider the complement-fixation test essential in controlling the susceptibility of experimental dogs.

The published results of Goret, *et. al.* show that some dogs displayed a diphasic thermal reaction and the majority developed diarrhoea. These clinical signs are more typical of distemper than of H.C.C. Some dogs did not die until as late as 17 to 43 days after inoculation suggesting that, if H.C.C. was responsible, it was contracted other than from the inoculum. Those who have worked with H.C.C. in the laboratory will know how difficult it is to account for some cases of natural spread. We feel, therefore, that the reported results indicating the reproduction of Rubarth's disease in dogs by the inoculation of material that had received a number of passages in ferrets, may be capable of an alternative explanation.

CONCLUSION

Attempts to adapt a number of strains of Rubarth's virus to ferrets using various routes of inoculation, various strains of virus and both English and French ferrets, all failed. Virus may have been present in very small quantity in a pair of ferrets five days after inoculation with dog material, but none could be recovered from the second passage in ferrets. We have been unable to confirm the presence of the virus of Rubarth's disease in material that had received 6 to 22 passages in ferrets in France.

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THE DIFFERENTIAL LEUCOCYTE COUNT IN THE NORMAL PIG

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INTRODUCTION

In connection with an investigation on tuberculous sensitivity in the pig it was necessary to collect information on the total white cell and differential white cell counts in the normal pig. In the available literature there were few references to this aspect of the haematology of the pig. Scarborough (1931) summarised 140 counts by various authors of the total white cells and also the differential leucocyte counts. The ages of the pigs were not stated. Frazer (1938) recorded counts for pigs at 1 to 3 days, 7 to 28 days and 2 to 7 months while Giltner (1907) also has recorded counts for pigs 10 weeks old. Venn (1944) estimated average white cell counts for two litters between the ages of 5 and 84 days. The figures for these authors are summarised in Table 1.

Table I

Total and Differential White Cell Counts in the Pig

			Percentage				
Author	*Age*	*Total Count*	*Poly-morphs*	*Eosino-phils*	*Baso-phils*	*Lymph.*	*Mono.*
Giltner (1907)	10 wks.	18,000	33·8	3·4	0·4	60·0	2·4
Scarborough (1931)	—	15,800	39·0	4·5	1·2	52·1	3·3
Frazer (1938)	2 7 mths.	21,000	39·9	5·5	0·5	48·0	6·1
Venn (1944)	84 days	15,980	42·5	2·9	0	52·8	1·8

In the present investigation the total white cell count and the percentage and absolute number of lymphocytes, polymorphonuclears and eosinophils were calculated.

METHODS

A series of 110 bloods was examined comprising 30 bacon pigs, 30 sows and 50 pigs within the age range 8 to 16 weeks. The blood samples from the sows and bacon pigs were collected at Belfast Abattoir between 10 to 11 a.m. The pigs had been rested and fasted over-night. The pigs were driven singly into a slaughter pen, stunned electrically and the jugular vein severed. There was no obvious excitement during the process of slaughter. The blood samples were collected on a slide from the incised jugular vein. A blood film was made immediately and at the

same time a sample was diluted with 2 per cent acetic acid for the total white cell count. Each diluted sample was stored in a labelled glass tube pending the count being completed. The total white cells were estimated in all cases within one hour of the blood being diluted with the acetic acid.

In the case of the 8 to 16 week old pigs these samples were collected on various occasions from the laboratory herd. This group comprised 20 pigs bled at 11 a.m. and 30 pigs bled at 4 p.m. The morning group were bled 2 to 3 hours after feeding while the afternoon group were bled before feeding. The marginal ear vein was punctured and sufficient blood was lifted on a clean slide to make a suitable film. For the total white cell counts blood was drawn directly into the diluting pipette, diluted and stored as in the case of the abattoir samples. In addition 53 blood samples from new born piglets were examined.

TABLE 2

SOWS EX BELFAST ABATTOIR. SAMPLES TAKEN 10—11 A.M.

Pig No.	*Total White Cell Count*	*Differential Count*					
		Polymorphs		*Lymphocytes*		*Eosinophils*	
		No.	%	*No.*	%	*No.*	%
1	13,000	4,240	32	8,800	68	0	—
2	13,400	3,730	28	9,360	76·5	67	0·5
3	10,200	2,310	22	8,190	77·5	51	0·5
4	14,400	2,500	19	11,600	81	0	—
5	14,750	5,040	35	8,960	64	147	1
6	16,350	4,800	29	11,200	70	163	1
7	14,150	3,940	27	9,800	70	443	3
8	14,900	3,600	24	11,400	76	0	—
9	13,650	5,130	38	8,370	62	0	—
10	12,550	3,000	24	9,000	72	500	4
11	11,050	6,160	55·5	4,620	44	55	0·5
12	17,350	7,140	43·0	9,520	56	173	1
13	18,150	6,120	34·5	11,520	65·5	0	—
14	20,900	7,140	35	13,800	65	208	1
15	12,650	4,500	36	8,000	64	0	—
16	21,550	7,980	38·5	12,600	61·5	0	—
17	19,950	5,850	31	13,100	69	0	—
18	17,450	6,800	39	9.800	56	870	5
19	18,350	4,810	27	12,950	70	549	3
20	13,350	4,860	36	8,100	59	665	5
21	18,850	6,840	38	10,240	57	910	5
22	13,850	4,050	31	8,370	62	966	7
23	20,300	6,720	33	13,200	63	812	4
24	13,300	4,410	35	8,640	64	133	1
25	24,950	9,500	39	12,750	51	2,490	10
26	14,150	4,760	33	9,240	67	0	—
27	25,550	10,400	40	15,150	60	0	—
28	11,350	4,840	22	7,010	73	565	5
29	19,550	6,840	35	12,430	64	195	1
30	17,100	7,820	46·5	8,500	50	513	3·5
Average	15,900	5,593	35	9,974	63	318	2
Variation	10,200—25,550	3,310—10,400		4,620—15,150		0—966	

Total White Cells. A Neubauer improved type haemocytometer was used and the cells in 4 groups of 16 small squares were counted. These multiplied by 50 gave the total number of cells per c.mm.

Differential Count. Films were stained by the rapid Giemsa method. They were fixed and stained in the concentrated solution for two minutes. An equal amount of distilled water was added and the slide tilted to mix water and stain. Staining was continued for a further three minutes. The stain was then flushed off under the tap and the film differentiated with distilled water (pH 6.8) until it turned pink. In all cases 200 cells were counted. This technique was found adequate for the purpose in view.

RESULTS

Sows. The ages of the sows were unknown but it is unlikely that they were under three years old, and most of them had proved un-

TABLE 3

BACON PIGS EX BELEAST ABATTOIR. SAMPLES TAKEN 10-11 A.M.

Pig No.	*Total White Cell Count*	*Differential Count*					
		Polymorphs		*Lymphocytes*		*Eosinophils*	
		No.	%	*No.*	%	*No.*	%
1	16,200	4,800	30·5	10,840	69	81	0·5
2	15,400	4,650	29·5	10,850	69	210	1·5
3	13,200	4,420	34	8,250	65·5	66	0·5
4	13,800	5,320	39	8,400	61	0	—
5	12,000	2,640	22	9,360	78	0	—
6	10,500	2,200	21	7,560	71	840	8
7	12,500	3,350	27	9,000	72	125	1
8	14,500	2,320	16	12,180	84	0	—
9	12,000	4,200	21	5,910	71	500	5
10	10,000	1,900	19	7,720	75	600	6
11	9,400	3,780	42	5,400	58	0	—
12	18,800	6,660	36	11,840	64	0	—
13	16,200	5,910	37	9,920	63	0	—
14	9,800	3,250	34	6,380	66	0	—
15	10,600	4,620	44	6,050	66	0	—
16	10,800	3,780	36	7,120	64	0	—
17	11,200	3,740	34	7,160	66	0	—
18	11,400	4,600	41	6,700	59	0	—
19	18,700	3,900	20	14,100	79	187	1
20	14,300	3,100	23	11,200	77	0	—
21	17,100	4,760	29	11,900	70	171	1
22	13,400	4,860	35	8,500	63	268	2
23	14,900	3,300	22	11,600	78	0	—
24	14,000	3,300	23	10,200	73	560	4
25	13,550	2,720	21	10,800	79	0	—
26	11,100	3,420	31	7,700	69	0	—
27	8,900	1,780	20	7,020	79	89	1
28	19,200	4,090	23	14,440	76	192	1
29	21,350	4,200	20	16,800	80	0	—
30	19,600	4,940	26	14,060	74	0	—
Average	13,747	3,857	28	9,672	71	137	1
Variation	8,900—21,350	1,780—6,660		5,400—16,800		0—840	

satisfactory as breeders. In this group the total white cell count varied from 10,200 to 25,550 cells per c.mm. with an average of 15,900 over the 30 specimens. With one exception the total polymorphonuclear leucocytes were less than the total lymphocytes. The exception noted was a sow which showed 55·5 per cent. polymorphonuclears to 44 per cent lymphocytes. Five out of the 30 sows showed no eosinophils in the 200 cells counted. (Table 2).

Bacon Pigs. A series of blood samples from bacon pigs was also examined. These pigs were about 8 to 9 months old. The total white cells in this series varied from 8,900 to 21,350 per c.mm. with an average of 13,747. Nine of the bacon pigs showed no eosinophils in the 200 cells counted. (Table 3).

TABLE 4

PIGS 8–16 WEEKS OLD EX LABORATORY HERD. SAMPLES TAKEN AT 4 P.M.

Pig No.	*Total White Cell Count*	*Differential Count*					
		Polymorphs		*Lymphocytes*		*Eosinophils*	
		No.	%	*No.*	%	*No.*	%
1	21,400	5,460	26	15,120	72	428	2
2	16,200	4,170	26	11,520	72	324	2
3	21,000	6,520	31	14,480	68·5	105	0·5
4	25,200	5,750	23	18,750	75	504	2
5	27,400	7,200	27	18,800	70	812	3
6	14,400	2,800	20·5	22,620	79	72	0·5
7	13,200	5,200	39·3	7,200	57	396	3·3
8	14,200	2,840	20·5	10,920	78·5	142	1
9	14,800	8,600	18·5	11,600	80	222	1·5
10	15,000	5,100	37	9,300	62	150	1
11	16,800	4,200	25	11,930	73	336	2
12	13,400	3,120	23	9,920	73	402	3·5
13	15,200	4,600	27·5	10,540	72·5	0	—
14	28,000	9,140	33·5	17,620	63·5	560	2
15	23,400	7,140	31·5	15,420	67·5	352	1·5
16	35,400	6,250	16·5	28,450	81·5	708	2
17	17,200	4,420	25·5	12,130	74	86	0·5
18	18,400	4,610	25	13,320	72	552	3
19	15,200	2,210	14	12,840	85	152	1
20	16,200	3,840	24	11,100	70	972	6
Average	19,150	4,869	25·4	13,810	72·7		1·9
Variation	13,200— 35,400	2,210— 9,140		7,200— 28,450		0—972	

Young Pigs. Fifty blood samples from pigs 8 to 16 weeks old were examined. The animals were divided into the two lots, 20 being bled in the afternoon and 30 in the morning. The 20 samples taken at 4 p.m. gave an average total white cell count of 19,150 with a variation in individual cases of from 13,200 up to 35,400 per c.mm. In only one pig in this group were no eosinophils recorded in 200 cells

counted. Among the 30 pigs of the group bled at 11 a.m. total white cell counts averaged 30,666 varying from 20,500 to 43,250 per ml. No eosinophils in 200 cells was recorded in five animals in this group. (Tables 4 and 5).

Newly Born Piglets. In the 53 samples taken at birth the total white blood cell count averaged 11,783, the lowest being 5,850 and the highest 20,800. The polymorphonuclears averaged 62 per cent. and the lymphocytes 38 per cent. In 17 of the samples, 1 to 2 per cent of eosinophils were recorded. After birth the total white cell count begins to rise. There is an initial fall in the number of polymorphonuclears during the first 7 to 10 days and thereafter an increase. The number of lymphocytes increases over the first few weeks. The net result of these changes brings the cellular content

TABLE 5

PIGS 8-16 WEEKS OLD EX LABORATORY HERD. SAMPLES TAKEN AT 11 A.M.

Pig No.	*Total White Cell Count*	*Differential Count*					
		Polymorphs		*Lymphocytes*		*Eosinophils*	
		No.	%	*No.*	%	*No.*	%
21	27,400	11,340	41	15,660	58	274	1
22	25,150	8,250	33	15,500	62	1,506	6
23	41,800	14,580	35	26,800	64	418	1
24	25,100	10,250	41	14,000	56	502	2
25	32,300	9,820	31	22,140	69	0	—
26	27,750	9,760	35	17,620	64	277	1
27	31,900	8,740	27	22,200	69·5	480	3·5
28	26,350	10,610	41	15,080	58	263	1
29	25,850	10,850	41	15,010	58	258	1
30	22,700	7,260	33	15,380	67	0	—
31	28,000	9,520	34	17,910	64	560	2
32	43,250	17,200	40·5	24,180	56·5	1,297	3
33	26,800	8,320	31	17,280	64	1,340	5
34	25,350	8,740	34·5	16,260	65	126	1·5
35	32,400	13,680	43	17,920	56	324	1
36	30,200	14,400	48	15,780	50·5	151	0·5
37	32,150	12,520	35	18,560	58	1,284	4
38	34,000	13,150	38	21,350	61	346	1
39	24,350	11,040	46	12,680	53	243	1
40	37,600	13,480	36	23,260	61	418	3
41	30,500	11,750	39	18,300	61	0	—
42	20,500	6,150	31	13,650	65	820	4
43	23,400	9,350	40	13,550	58	468	2
44	30,850	9,920	31·5	21,200	68	154	0·5
45	42,200	17,640	41	24,369	58	422	1
46	37,650	14,420	38	23,360	61	376	1
47	28,100	12,100	43	15,880	57	0	—
48	32,400	11,210	35	20,690	64	162	0·5
49	29,600	13,100	43	16,340	55	592	2
50	24,200	9,820	41	14,220	59	0	—
Average	30,666	11,132	36·3	18,850	62	489	1·6
Variation	22,700—43,250	6,150—17,640		13,650—26,800		0—1,506	

and the numerical relationship between the lymphocytes and polymorphonuclears into line with the counts in the blood of older pigs. These figures agree fairly closely with those recorded by Venn(1944) for very young pigs.

The day to day variations in the white cell content of the blood in individual pigs was also investigated. In Table 6 details are given of the daily variations shown by one of this group over a period of 15 days. In addition, some observations were made on the changes seen when blood samples were taken at intervals, of two hours. Even in such cases marked variations may be encountered and successive counts of 47,400 and 19,950 have been recorded for total white cells in the same pig.

TABLE 6

PIG NO. Z24, 14 WEEKS OLD. DAILY BLOOD COUNTS TAKEN AT 10 A.M.

Day No.	*Total White Cell Count*	*Differential Count*					
		Polymorphs		*Lymphocytes*		*Eosinophils*	
		No.	%	*No.*	%	*No.*	%
1	48,800	14,250	28	34,300	70	488	1
2	41,050	16,650	41	22,140	54	2,050	5
3	49,950	21,400	43·5	26,100	52·5	1,996	4
4	31,300	11,460	37	18,850	61	616	2
5	36,600	16,180	44	19,140	52	1,464	4
6	22,800	7,560	33	15,160	66	288	1
7	34,600	10,750	31	23,000	66	1,038	3
8	49,550	22,400	45	26,300	53	990	2
9	37,550	11,250	30	23,900	65	1,875	5
10	42,600	18,720	43·5	23,100	54·5	852	2
11	35,200	14,350	41	19,600	55	1,408	4
12	36,700	8,680	23·5	26,640	72·5	1,468	4
13	37,200	8,870	24	26,440	71	1,850	5
14	38,200	10,940	29	26,100	68	1,146	3
15	31,950	8,660	27	23,100	72	319	1
Average	38,270	13,475	35	23,590	61·5	1,121	3·5
Variation	22,800—49,950	8,660—22,400		15,160—34,300		319—2,050	

Range of Variation.

The range of variation in the cell counts for total white cells, polymorphonuclears and lymphocytes was comparable between the sow and bacon pig groups. In the store pigs (8 to 16 weeks old) the total white cell count was higher compared with the older pigs and within this group the morning bleeding gave an average total white cell count of 30,666 as compared with an average of 19,150 for the afternoon bleeding. It is interesting to note that the average polymorphonuclear percentage at the morning was 36 per cent. whereas this figure fell to 25 per cent. at the afternoon bleedings. There was

a complementary variation in the lymphocytes. Little change occurred in the eosinophils. The time of feeding may be of some significance in these changes in the cellular content of the blood between morning and afternoon samples.

The overall increase in the total white cells in the store pig group may in part be a function of the age of the pig and in part associated with excitement at the time of bleeding. The catching and restraining of the pigs in this group for the purpose of collecting the blood inevitably cause considerable struggling. Smaller pigs are picked up and held under the arm of an assistant while the blood is drawn but as the pig gets older this method is not applicable; such pigs are caught and a twitch is placed round the upper jaw behind the canine teeth. Pigs that are bled regularly may become relatively amenable to restraint but there are others that continue to struggle fiercely even though they may be bled regularly over a period of several months. This excitement may be a factor in causing the wide range in the total white cells though we have not been able to find any co-relation in practice between the degree of struggling and any extreme variation in the total white cell count. This excitement factor was not in evidence in the older pigs bled at the abattoir.

In the 110 samples examined the total white cell count averaged 19,866 with 6,363 polymorphonuclears (32 per cent) 13,076 lymphocytes (66·4 per cent) and 327 eosinophils (1·6 per cent). (Table 6).

TABLE 7

AVERAGE CELL COUNT OVER 110 NORMAL BLOOD SAMPLES

	Total White Cell Count	*Polymorphs*		*Lymphocytes*		*Eosinophils*	
		No.	%	No.	%	No.	%
Sow Group	15,900	5,593	35	9,974	63	318	2
Bacon Group	13,747	3,857	28	9,672	71	137	1
Store Pigs Morning	30,666	11,132	36	18,850	62	489	1·6
Store Pigs Afternoon	19,150	4,869	25	13,810	73	363	1·9
Average	19,866	6,363	32	13,076	66·4	327	1·6

Serial Blood Counts at Weekly Intervals

As a preliminary step to studying the effect of the tuberculin test on the blood picture, three pigs were bled at weekly intervals over a period of 16 weeks, starting when the pigs were 10 weeks old. The results in two animals of this group are depicted in Fig. 1. With the exception of the first bleeding and that at the fourteenth week all the bleedings were done between 10 and 11 a.m. On the first and fourteenth week the blood was drawn at 3 p.m. It will be seen that

the total white cell count in the individual pig varies within fairly wide limits. This variation may be reflected mainly in the lymphocytes at one bleeding or in the polymorphonuclears at another bleeding. In Pig Z.28, for example, over the 16 week period the total white cells varied from 19,200 to 48,550 cells per c.mm. the percentage of polymorphonuclears varied from 16 per cent to 40·5 per cent and the lymphocytes varied from 56·5 per cent to 83·5 per

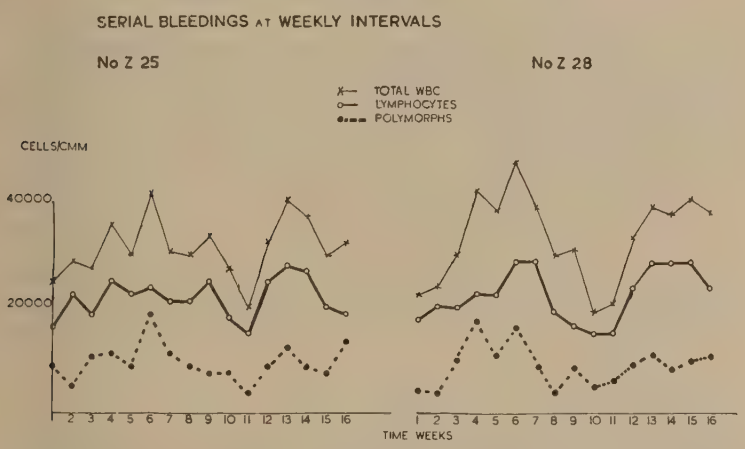

FIG. NO. 1

Variations in the white cell count in normal pigs over a period of 16 weeks

cent. Over the same period the eosinophils varied from zero to 5 per cent. However, in spite of the wide variation shown in the total white cell counts at different bleedings, the numerical relationship between the lymphocytes and the polymorphonuclears remained fairly constant.

The evidence from the observations recorded here indicates that in the normal pig the total white blood counts vary considerably from day to day and even from hour to hour. Normally the lymphocytes are consistently more numerous than the polymorphonuclears, except in the new born pig where a comparative lymphopenia is present. A small number of eosinophils may be present at birth, but the average eosinophil content in the blood of even the older pigs remains low, being of the order of 1 to 3 per cent. Exceptionally an animal may register a eosinophil count of 8 per cent. There is a possibility that in these cases a recent exposure to infection with Ascaris lumbricoides may be the cause of this comparative eosinophilia.

Oldham and White (1944) reported on the blood picture in pigs which had been fed large doses of ascarid eggs. In the control pigs and in the experimental pigs at the time of feeding the eggs the

eosinophil count averaged 2 to 3 per cent. Animals given a single large dose of eggs showed an eosinophilia reaching its maximum in 16 to 20 days. Up to 23·7 per cent. of eosinophils was recorded in one case. Where repeated doses of eggs were administered over a period, the eosinophilia was induced to the initial dose only and the level returned to normal after 4 to 6 weeks.

SUMMARY

Total white cell and differential white cell counts are given for different age groups of pigs. Normally the number of lymphocytes is consistently fewer than that of the polymorphonuclears. Blood counts at weekly intervals show wide variations in the total white cells from week to week. In the new born pig a comparative lymphopenia is present.

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EXPERIMENTAL JOHNE'S DISEASE IN CATTLE

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INTRODUCTION

Many aspects of research on Johne's disease have been handicapped not only by the lack of a suitable laboratory animal but also by the uncertainty attendant upon the experimental reproduction of the disease in cattle, which has led to the use of naturally occurring clinical cases of the disease for purposes of research. Such animals were not entirely satisfactory but the difficulty of producing artifical infection with some degree of certainty was not completely overcome in the past; consequently, unspecified amounts of culture have been given to cattle by different routes with varying degrees of success, while the even more haphazard method of feeding minced infected intestinal mucosa has also been used.

This paper describes an attempt to find a reproducable method of inducing Johne's disease in experimental cattle and a study of the disease so caused by means of examination of faeces and at autopsy.

METHODS

Experimental animals. Twelve Ayrshire bull calves bred at this Field Station, from attested herds with a history of very low incidence of Johne's disease, were taken from their dams at parturition before they had sucked. From birth, until the end of the experiment between two and three years later, they were kept in pairs in loose boxes in strict isolation. During the first 48 hours of life they received their dam's colostrum, subsequently bulk attested raw milk, all of which was bucket fed. They were weaned gradually to concentrates and hay, and eventually received about 2 lbs. of concentrates per day with hay and water ad lib; no green food was given at any time. The calves were castrated when approximately three months old; at no time during the experiment did they show signs of intercurrent disease and worm egg counts invariably gave insignificant results.

Infection. A mixture of five recently isolated strains of *Mycobacterium johnei* was used in an arbitrary dose of 100 mg. to infect each calf. None of the strains had been subcultivated more than four times since their primary isolation and none grew without the addition of heat-killed *M. phlei* to the medium. They were grown separately for 6 to 8 weeks on Dunkin's medium, when the growth was removed, weighed wet and ground in an agate mortar with Ringer solution so that the final suspension contained a mixture of

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TABLE 1

THE EXPERIMENTAL INFECTION OF CALVES WITH *M. Johnei*

Volume and Route of Injections

Number	*Age Infected*	*Route*	*Dosage*
1 and 2	Birth	Per os	100 mg. in 1 ml.
3 and 4	Birth	I.V.	100 mg. in 10 ml.
5 and 6	3 Months	Per os	100 mg. in 1 ml.
7 and 8	3 Months	I.V.	100 mg. in 10 ml.
9 and 10	6 Months	Per os	100 mg. in 1 ml.
11 and 12	6 Months	I.V.	100 mg. in 10 ml.

all five strains in equal parts by weight. The calves were infected as shown in Table 1; those infected per os received the suspension as far back on the tongue as it could be placed with a pipette; the remainder were inoculated into the jugular vein.

Tuberculin tests. The calves were tested every three months after infection. P.P.D. avian and mammalian tuberculin and P.P.D. johnin, all in strengths of 0·4 mg. P.P.D. per ml., and received through the courtesy of Dr. H. H. Green, Weybridge, were used in a single intradermal injection in the side of the neck. The reactions were measured after 72 hours.

Cultural examination of faeces and tissues. The methods and medium used in preparing cultures of *M. johnei* in this laboratory have already been described in detail elsewhere (Taylor, 1950). Briefly, faeces are treated with antiformin and tissue with oxalic acid prior to inoculation on at least five bottles of medium. Initially, Dunkin's medium was used, but towards the end of the experiment and throughout the post-mortem examinations, an egg yolk medium containing 4 per cent of glycerine and about 1 per cent of heat-killed *M. phlei* was used. The bottles were incubated for three months before being discarded as negative, but colonies resembling those of *M. johnei* were subcultivated to plain egg, glycerine egg and the *phlei* medium. Cultures of small acid-alcohol-fast bacilli which grew only on the last named medium after from four to six weeks incubation at 37°C., were accepted as *M. johnei*.

Cultures were prepared from the faeces of each calf every month after infection. At autopsy a number of tissues were examined.

In the alimentary tract, if lesions were present, cultures were made from the abomasum, duodenum, jejunum, ileum, ileo-caecal valve and large bowel. When lesions were not obvious, the small intestine was divided into approximately 8 ft. lengths from each of which a number of pieces of mucous membrane were removed and mixed prior to grinding with oxalic acid. Thus, from "no lesion" cases fifty to sixty bottles of medium were used for the small intestine alone, apart from the routine cultures from other parts of the bowel. In addition, from each animal cultures were made from the liver, lungs and spleen, from the submaxillary, retropharyngeal, broncho-mediastinal, hepatic and ileo-caecal lymph nodes, and occasionally from the prescapular and iliac lymph nodes.

RESULTS

All the experimental animals were destroyed for examination at intervals between two and three years after infection; none actually died from Johne's disease. For ease of description the experimental results have been grouped under four headings—clinical signs, examination of faeces, tuberculin tests and post-mortem findings.

Clinical Signs.

The clinical syndrome of Johne's disease was eventually shown by four of the twelve animals, Nos. 1, 3 and 4 infected at birth, and No. 7 infected when three months of age. These animals progessed normally, with the exception of No. 3, which was slightly under-sized, until about 12 to 15 months after infection, when occasional bouts of diarrhoea, lasting for one or two days, made their appearance. The disease then followed its usual insidious course, with gradual loss of condition and more frequent diarrhoea, until the animals were aged about two years, when they had developed into typical clinical cases of Johne's disease, except that the diarrhoea was never so profuse as the bodily condition might have warranted. Had green food or roots been fed, however, it is possible that the classical type of profuse fluid diarrhoea would have appeared.

The disease in Nos. 1, 4 and 7 continued its usual course, but in No. 3, at the age of 23 months, suddenly became acute and the animal was destroyed. This bullock had shown well marked loss of condition and intermittent diarrhoea for roughly six months, but on 15th June, 1947, did not feed and on the following day diarrhoea was profuse. On June 17th, the faeces were fluid, black in colour with blood clots and much mucus giving to the tail and hind-quarters a black, sticky appearance. The animal was very weak, most unwilling to rise and showed a temperature approaching 103°F. Loss of condition was very rapid; the black diarrhoea with fresh blood and blood clot continued and the animal was destroyed on June 19th. The post-mortem findings are described later.

The remaining eight animals continued to thrive until the end of the experiment when most of them were in good store condition.

TABLE

CULTURAL EXAMINATION OF FAECES OF

Nos.		*Age in*														
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
1	J	+	—	+	—	+	—	+	—	+	—	+	+	+	+	+
2	J	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	J	—	+	+	—	+	—	—	—	+	+	—	+	+	+	+
4	J	+	—	—	+	—	+	—	+	+	+	+	+	+	+	+
5				J	—	—	—	—	—	—	—	—	—	+	—	—
6				J	—	—	—	—	—	—	—	—	—	—	+	—
7				J	—	—	—	—	—	—	—	+	+	+	+	+
8				J	—	—	—	—	—	—	—	—	—	+	—	—
9							J	—	—	—	—	—	—	—	—	—
10							J	—	—	—	—	—	—	—	—	—
11							J	—	—	—	—	—	—	—	—	—
12							J	—	—	—	+	—	—	—	—	—

+ = *M. johnei* recovered
J = Infected.
D = Destroyed.

Examination of Faeces

The result of the cultural examination of faeces from each animal at monthly intervals after infection is shown in Table II. The isolation of *M. johnei* in culture from lightly infected faeces may, however, be so adversely affected by a number of different factors that it is extremely likely that on many occasions the organism was not recovered when it was indeed present in the original material.

Three of the four calves infected at birth began to excrete the organism in their faeces more or less immediately, in contrast to the "three months" group from which no isolation was obtained until eight months after infection. From the "six months" group *M. johnei* was recovered but once and that from an animal in which, as will be shown subsequently, no organisms were found at autopsy.

Microscopic examination of the faeces was of little value in following the progress of the infection. Films were prepared both directly, by emulsifying a loopful of faeces with normal saline on a slide, and after concentration with antiformin, which has an unfortunate tendency to break up any clumps of organisms that may

II

ARTIFICIALLY INFECTED CATTLE FOR *M. johnei*

Months																
16	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	*31*	*32*
+	+	+	+	+	+	+	+	+	D							
—	+	—	+	+	—	+	+	+	—	+	D					
+	+	+	+	+	+	+	D									
+	+	+	+	+	+	+	+	+	+	+	+	D				
—	—	—	+	+	—	—	+	—	+	—	D					
—	—	—	+	—	—	—	—	+	—	+	—	D				
+	+	+	+	+	+	+	+	D								
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	D	
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	D
—	—	—	—	—	—	—	—	—	—	—	—	—	—	D		
—	—	—	—	—	—	—	—	—	—	—	—	D				
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	D

in culture

be present. Single organisms were seen repeatedly throughout the experiment, but not until clinical changes were beginning to become obvious did the diagnostic groups and clumps of bacilli appear in the films.

Tuberculin Tests

The result of tuberculin and johnin tests performed every three months after infection is shown in Table III. These figures are not comparable with those obtained in the field nowadays in this country, as it should be borne in mind that they were begun in 1945, before the introduction of the "single" intradermal test as the official test, and that "equal strength" P.P.D. tuberculins were used, i.e. all contained 0·4 mg. P.P.D. per ml., whereas the official tuberculin now contains 0·5 mg. of avian P.P.D. and 2·0 mg. per ml. of mammalian P.P.D. respectively. Consequently, the reactions shown in the table particularly those to mammalian are smaller than might be expected with the present strength of tuberculin. The table does

TABLE III

TUBERCULIN AND JOHNIN TESTS ON CATTLE EXPERIMENTALLY INFECTED WITH *M. johnei*

The figures express the increase in skin thickness at the 72nd hour after injection

Number		0	3	6	9	12	15	18	21	24	27	30
						Age in months						
1	A		1	6	5	4	9	9	3	6		
	M	I	0	1	1	1	0	1	1	0		
	J		2	0	2	2	4	2	1	0		
2	A		1	10	12	10	14	7	4	7		
	M	I	0	0	2	1	1	1	1	2		
	J		0	4	7	7	11	5	3	4		
3	A		1	5	9	6	1	6	3			
	M	I	0	1	1	1	1	1	1			
	J		0	0	4	1	1	1	2			
4	A		7	13	9	8	8	6	3	2	4	5
	M	I	2	2	1	0	2	0	0	2	1	0
	J		2	1	3	2	2	0	1	1	1	1
5	A			6	3	1	17	14	1	6		
	M	I		1	1	0	2	3	0	0		
	J			1	0	1	13	7	4	4		
6	A			3	2	2	1	2	6	8	10	
	M	I		1	0	0	1	0	2	0	1	
	J			3	0	1	0	2	4	4	6	
7	A			2	14	11	6	6	4			
	M	I		0	2	1	0	0	1			
	J			1	8	4	3	3	4			
8	A			3	9	7	6	3	0	1	3	3
	M	I		0	1	0	3	2	0	1	0	0
	J			1	3	1	2	3	0	1	1	0
9	A				1	1	2	1	2	2	1	4
	M			I	0	0	0	0	0	1	0	0
	J				1	1	3	0	1	1	1	3
10	A				1	1	2	1	2	8	4	
	M			I	1	0	1	1	1	1	2	
	J				1	0	1	1	4	8	4	
11	A				12	2	3	3	3	1	1	
	M			I	0	1	0	2	0	1	0	
	J				4	4	2	1	0	1	1	
12	A				4	6	5	1	0	1	0	1
	M			I	0	0	0	1	1	0	1	0
	J				1	1	2	2	0	0	1	3

A = avian P.P.D. tuberculin M = mammalian P.P.D. tuberculin J = P.P.D. johnin I = infected

show, however, a superiority of P.P.D. avian tuberculin to P.P.D. johnin in eliciting a reaction in infected animals and also the fact that the allergic response is usually at its height during the earlier stages of the infection and tends to fade out with the onset of clinical signs of the disease.

Post-mortem Findings

The result of the post-mortem examinations is shown in Table IV, which indicates that of the twelve calves, clinical disease developed in four, Nos. 1, 3, and 4 infected at birth and No. 7 infected when three months old. The remaining animals were in good condition when slaughtered but typical lesions of the disease were present in No. 2, infected at birth, and Nos. 5 and 6 infected when 3 months old. Very small numbers of *M. johnei* were isolated in culture from two animals, Nos. 8 and 10, in which lesions of the disease were not found either macroscopically or histologically, whereas in the remainder, Nos. 9, 11 and 12, injected when 6 months old, infection was not demonstrated.

Calf No. 3, developed the disease in an acute form after having shown clinical symptoms for some months and was destroyed. At autopsy there was an almost complete absence of fat and lesions were confined to the alimentary tract. The abomasum showed a few petechial haemorrhages and gross lesions of Johne's disease were present from the pylorus to the anus. The small intestine was almost empty but the last 15 to 20 feet were abnormally thickened and showed bright red blood in the folds of mucous membrane, the contents being an adherent mixture of blood and mucus. The terminal portion of the ileum showed definite ulceration, which was confirmed histologically, a number of eroded areas, up to 2 cm. in diameter being present. The appearance of the large intestine was like that of the ileum; there was fresh blood in the folds of intestine and the contents were similar. A shallow irregularly edged ulcer, approximately 3 cm. across, was present in the caecum near the valve. The mesenteric lymph nodes were in many cases swollen and oedematous. It is shown in Table IV, that *M. johnei* was recovered from all the tissues from which cultures were made; in fact an almost confluent growth was obtained from tissues such as the liver and lungs, so that a condition resembling bacteraemia may have been present in this animal.

When it became apparent from these experimentally infected cattle showing clinical symptoms of Johne's disease, that *M. johnei* might be isolated in culture from tissues other than those of the alimentary tract, a number of naturally occurring cases of the disease were examined with the result shown in Table V. This indicates that, from clinical cases of Johne's disease in cattle *M. johnei* may be regularly isolated not only from the intestine and its associated lymph nodes, but also from many of the other tissues of the body.

TABLE

POST-MORTEM EXAMINATION OF CATTLE

Number	Age Infected in months	Clinical Signs	Macroscopic lesions	Age in Months	RECOVERY OF Abomasum	Small Intestine	Large Intestine	Liver	Spleen
1	0	YES	YES	25	—	+	+	+	—
2	0	NO	YES	26	ND	+	—	—	—
3	0	YES	YES	22	+	+	+	+	+
4	0	YES	YES	34	+	+	+	+	—
5	3	NO	YES	26	—	+	—	—	—
6	3	NO	YES	29	—	+	—	+	—
7	3	YES	YES	24	—	+	+	+	—
8	3	NO	NO	34	—	1 +	—	—	—
9	6	NO	NO	32	ND	—	—	—	—
10	6	NO	NO	30	ND	—	—	—	—
11	6	NO	NO	28	ND	—	—	—	—
12	6	NO	NO	31	ND	—	—	—	—

+ = *M. johnei* recovered in culture
— = *M. johnei* not recovered
1 = 1 colony: 2 = 2 colonies
ND = Not done.

IV

ARTIFICIALLY INFECTED WITH *M. Johnei*

M. JOHNEI *IN CULTURE*

Lung	*Mesenteric Lymph node*	*Hepatic lymph node*	*Bronchomediastinal lymph node*	*Submaxillary lymph node*	*Retropharyngeal lymph node*	*Iliac l. node*	*Prescapular lymph node*
+	+	+	+	+	+	ND	ND
—	+	—	—	—	—	ND	ND
+	+	+	+	+	+	ND	+
+	+	+	+	+	+	ND	+
—	+	—	—	—	—	ND	ND
—	+	+	+	—	+	ND	ND
+	+	+	+	—	+	—	ND
—	+	—	—	—	—	ND	ND
—	—	—	—	—	—	ND	ND
—	+[2]	—	—	—	—	ND	ND
—	—	—	—	—	—	ND	ND
—	—	—	—	—	—	ND	ND

TABLE

RECOVERY OF *M. johnei* IN CULTURE FROM NATURALLY

Number	*Small Intestine*	*Large Intestine*	*Liver*	*Spleen*	*Lung*	*Mesenteric lymph node*
A.88	+	+	+	—	—	+
A.90	+	+	+	+	—	+ + T.B.
W.1	+	+	+	+	+	+
662	+	+	+	+	+	+
J.52	+	+	+	+	+	+
C.2	+	ND	+	+	+	+
J.140	+	+	+	ND	+	+
F.S.S.	+	ND	ND	ND	ND	+
49305	+	+	+	—	—	+
1418	+	+	+	—	+	+
452	+	+	+	—	—	+

+ = *M. johnei* recovered in culture.
— = *M. johnei* not isolated.

Occurring Clinical Cases of Johne's Disease

Hepatic lymph node	*Broncho-mediastinal lymph node*	*Submaxillary lymph node*	*Retro-pharyngeal lymph node*	*Iliac lymph node*	*Prescapular lymph node*
+	+	+	+	ND	ND
+	+	— + T.B.	+	ND	ND
+	+	+	+	ND	ND
+	+	+	+	ND	ND
+	+	—	+	ND	ND
+	+	+	+	ND	+
+	+	+	+	ND	ND
+	+	+	+	+	+
+	—	+	+	ND	ND
+	+	+	+	ND	+
+	+	—	—	ND	+

ND = Not

DISCUSSION

It has been recognised for many years that young cattle may be more susceptible to infection with *M. johnei* than older animals. This is well borne out by the experiments described in this paper, which have also established that a single inoculum of 100 mg. of culture of recently isolated strains will so infect the young calf that extensive lesions of the disease, if not always the complete clinical sydrome, may be reproduced. Older animals, i.e. those of 6 months of age, as was to be anticipated, were not so infected. It is conceivable that the young calf might be infected by a very much smaller inoculum than 100 mg. and that adult animals might also be infected by larger or repeated doses, but the primary purpose of the experiment, namely the determination of a suitable infecting dose, was achieved. There is no known method of assessing the virulence of individual strains of *M. johnei*; therefore, it would appear essential, in infection experiments, to use recently isolated strains of the organism. To establish with certainty that a bovine animal is free from infection with *M. johnei* is not practicable. The sheer bulk of the bovine intestine and its associated lymph nodes is so great that only a minute proportion may be examined culturally or microscopically and further, the difficulties inherent in the primary isolation of *M. johnei* militate against its demonstration when present in minimal numbers.

The number of animals used in this experiment was of necessity, small, but was sufficient to establish several features not previously recognised, or at least only suspected, regarding Johne's disease. Thus it has been confirmed that an infected animal, in apparently good health, may excrete the organism in the faeces for many months before either clinical signs become apparent, or infection can be demonstrated by microscopic examination of the faeces. This is of obvious importance in the control of the disease and it is unfortunate that the technical difficulties of the method, i.e. by culture from the faeces, are at present so great as to render it of little value as a routine method of diagnosis.

The tuberculin tests of these animals cannot properly be compared with present-day results, but again they tend to confirm an impression expressed elsewhere that allergic tests are of little value in the diagnosis of Johne's disease. (Taylor, 1952).

It has long been understood that in the animal body *M. johnei* was confined to the lesions of the disease in the intestine and to its associated lymph nodes. This opinion has rarely been disputed, except by Alexjeff-Goloff (1929) who "startled current opinion" (Levi, 1948) by recording the isolation of *M. johnei* from different organs and the milk, blood and urine of advanced cases of the disease. Other recorded instances of the isolation of the organism from extra-intestinal sites have been very few in number and were reviewed by Levi (1948) who was, however, able to obtain *M. johnei*

in culture twice from the pharyngeal lymph nodes and once each from the tonsil and spleen of naturally affected cows. The present work, however, has established that *M. johnei* may be regularly recovered in culture from almost any of the lymph nodes or abdominal or thoracic viscera of cattle showing clinical signs of the disease. Two distinct factors have probably been responsible for this result. First the organism is known to be resistant to destruction within the animal body and in clinical cases where it is usually present in large numbers in the mesenteric lymph nodes, it may escape from these into the lymph stream, from which a mild bacteraemia may be established. In the second place, the use of oxalic acid and an egg-yolk-phlei medium (Taylor, 1950) for the primary cultivation of *M. johnei* is greatly superior to methods previously used; in other words the technical methods employed by many of the earlier workers were insufficiently delicate to demonstrate organisms unless these were present in quantity, as in the intestine and mesenteric lymph nodes.

SUMMARY

The lesions and clinical syndrome of Johne's disease may be reproduced in cattle by the intravenous inoculation or oral administration of 100 mg. of recently isolated culture of *M. johnei* to young calves.

Calves so infected excrete *M. johnei* in their faeces for many months before clinical signs of the disease become apparent or the organisms can be demonstrated microscopically.

M. johnei may be recovered in culture, not only from the intestine and its associated lymph nodes but also from many of the other tissues of clinical cases of Johne's disease in cattle.

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THE EXPERIMENTAL INFECTION OF CATTLE WITH VARIETIES OF *MYCOBACTERIUM JOHNEI* ISOLATED FROM SHEEP

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INTRODUCTION

Taylor (1951) showed that one of three varieties of *Mycobacterium johnei* might be the cause of Johne's disease of sheep. The first is the classical type as isolated from Johne's disease in cattle, the second a strongly pigmented organism now known to occur in sheep in Scotland, the North of England and in Wales and the third is the cause of Johne's disease of sheep in Iceland. The Icelandic and pigmented strains differ from the classical type in their cultural requirements and neither have as yet been recorded as a cause of disease in cattle.

The work described in this paper was undertaken in an endeavour to confirm these ovine strains as varieties of *M. johnei* by examining their ability to produce the syndrome of Johne's disease in cattle.

MATERIAL AND METHODS

Experimental animals. Second generation Red Poll cross Ayrshire calves bred at this Field Station from an attested herd with no history of Johne's disease were used. They were all under the age of four weeks when infected and were thereafter maintained in loose boxes in strict isolation.

Inoculum. Six pigmented strains, none of which had been subcultivated more than six times since their primary isolation and three Icelandic strains also subcultivated six times were used; eight bovine strains recently isolated from apparently normal cattle (Taylor, 1952) served as controls. Each strain was grown separately for from six to eight weeks on egg-yolk-phlei medium (Taylor, 1950) when each group was mixed to give the three inocula ("pigmented", "Icelandic" and "bovine") and standardised in Ringer solution so that 2 mg. of culture was contained in each ml.

Infection. It was shown previously (Taylor, 1953) that Johne's disease may be reproduced in cattle by the intravenous inoculation of 100 mg. of culture to the young calf; this procedure was adopted in the present experiments. Three calves, Nos. P83, P84 and P87, each received 100 mg. of pigmented culture in a volume of 50 ml., two, Nos. P161 and P177, 100 mg. of Icelandic culture, and two Nos. P91 and P97, 100 mg. of bovine culture in a similar volume.

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Tuberculin tests. The comparative single intradermal tuberculin test using Ministry of Agriculture mammalian and avian P.P.D. tuberculin was applied to the calves every three months after infection.

Examination of faeces. Every month after infection the faeces were examined microscopically and culturally using antiformin and the egg-yolk-phlei medium.

Post-mortem examination. At autopsy the methods adopted were similar to those described previously (Taylor, 1953). Briefly, the extent of the lesions was noted and the presence of Johne's disease in the alimentary tract was confirmed microscopically and culturally. In addition, using oxalic acid and the egg-yolk-phlei medium, cultures were prepared from the liver, spleen and lungs and also from the following lymph nodes:—submaxillary, retropharyngeal, broncho-mediastinal, hepatic, ileo-caecal, and occasionally from the prescapular and the iliac. The bovine strains of *M. johnei* isolated from these tissues were identified by subcultivation to plain egg, glycerine egg, Dunkin's medium and the phlei medium. Small acid-alcohol-fast organisms which grew only on the last two media were accepted as *M. johnei.* The pigmented and Icelandic strains were identified by their ability to grow only on the egg-yolk-phlei medium.

RESULTS

The calves all progressed normally until they were approximately one year old, when symptoms of the clinical disease, i.e. intermittent diarrhoea, loss of condition and alteration in the coat, began to make their appearance. From fifteen to eighteen months after infection, all the calves, with the exception of No. P87, infected with the pigmented type, had become typical clinical cases of Johne's disease with emaciation and profuse diarrhoea. The calves infected with the Icelandic and bovine types excreted the organism continuously in their faeces from the first month after infection until the end of the experiment. The two calves which developed clinical symptoms, following infection with the pigmented type behaved similarly, but the organism was only recovered intermittently from No. P87 until the 13th month after infection. These results were obtained by cultural methods, for as has been shown previously (Taylor, 1953), it was impossible to confirm the presence of the disease by the microscopic demonstration of clumps of bacilli in the faeces until clinical signs were becoming evident, i.e. twelve to thirteen months after infection.

The result of the tuberculin tests on these animals is shown in Table I. The superiority of avian over mammalian tuberculin in evoking a reaction is well marked but such reactions are commonly met with in the routine tuberculin testing of cattle and can hardly,

TABLE I

TUBERCULIN TESTS

INFECTION	Calf No.	MONTHS AFTER INFECTION					
		3	6	9	12	15	18
PIGMENTED	P83	A 7	A 5	A 6	A 9	A 5	A 2
		M 3	M 0	M 1	M 3	M 3	M 0
	P85	A 6	A 8	A 9	A 6	A 3	A 2
		M 3	M 1	M 3	M 3	M 1	M 0
	P87	A 7	A 11	A 8	A 7	A 4	A
		M 3	M 1	M 1	M 2	M 2	M
ICELANDIC	P161	A 7	A 5	A 2	A 3	A 2	
		M 0	M 1	M 0	M 1	M 0	
	P177	A 8	A 9	A 4	A 2	A 3	
		M 1	M 2	M 1	M 0	M 1	
BOVINE	P91	A 6	A 4	A 4	A 4	A 4	
		M 3	M 0	M 2	M 1	M 2	
	P97	A 6	A 31	A 15	A 3	A 4	
		M 3	M 8	M 5	M 1	M 2	

The figures express the increase in skin thickness in mm. at the 72nd hour.

A=Avian PPD tuberculin

M=Mammalian tuberculin

in the present state of our knowledge, be regarded as specific for Johne's disease. This confirms an opinion expressed elsewhere (Taylor, 1952) that the comparative tuberculin test should be regarded as insufficiently specific to enable it to be of particular value in the diagnosis of this disease.

TABLE

RECOVERY OF *M. johnei* IN CULTURE

INFECTION	Calf No.	Small Intestine	Large Intestine	Liver	Lung	Spleen	Retropharyngeal lymph node
PIGMENTED	P83	+	+	+	+	—	+
	P85	+	+	+	+	+	+
	P87	+	+	—	—	—	—
ICELANDIC	P161	+	+	+	+	—	+
	P177	+	+	+	+	+	+
BOVINE	P91	+	+	+	+	+	+
	P97	+	+	+	+	+	+

+ = *M. johnei* recovered in culture
— = *M. johnei* not recovered in culture
ND = No cultures made

All the calves were slaughtered for post-mortem examination from sixteen to eighteen months after infection and all showed extensive lesions of Johne's disease in the alimentary tract. Even No. P87, which was in good store condition when slaughtered, showed well-marked changes in the small intestine and also early lesions in the large. The result of the cultural examination of these animals

II.

FROM EXPERIMENTALLY INFECTED CALVES

Suxmaxillary lymph node	*Broncho-mediastinal node*	*Hepatic lymph node*	*Ileo-coecal lymph node*	*Prescapular lymph node*	*Iliac lymph node*
+	+	+	+	—	—
+	+	+	+	ND	ND
—	—	—	+	ND	ND
—	+	+	+	ND	ND
+	+	+	+	+	ND
—	+	+	+	—	+
—	+	+	+	—	+

(Table II) confirms a previous observation (Taylor, 1953) that from clinical cases of Johne's disease the causal organism may be isolated not only from the alimentary tract but from many of the other tissues and lymph nodes of the body.

The disease in sheep caused by the pigmented type of *M. johnei* is characterised by the orange colour of the lesions in the intestine and

this phenomenon was very well shown in Nos P83 and P85 but to a less extent in P87. In these animals the mucous membrane of the affected parts of the intestine, although greatly thickened and showing the typical corrugations associated with the disease, was of a brilliant orange yellow colour. In the other animals the lesions were indistinguishable from those occurring in natural cases of Johne's disease.

The cultural characteristics of the pigmented and Icelandic strains were retained throughout the experiment, i.e. the organisms present in the faeces and in the tissues at autopsy grew only on the egg-yolk-phlei medium and not on Dunkin's medium. The bright orange colour of the colonies of the pigmented type was also unaffected.

SUMMARY

The pigmented and Icelandic varieties of *M. johnei* isolated from sheep have been shown to be capable of producing clinical Johne's disease in cattle.

Both varieties retained their characteristic cultural requirements and the orange colour of the pigmented type was unaffected after one passage through cattle.

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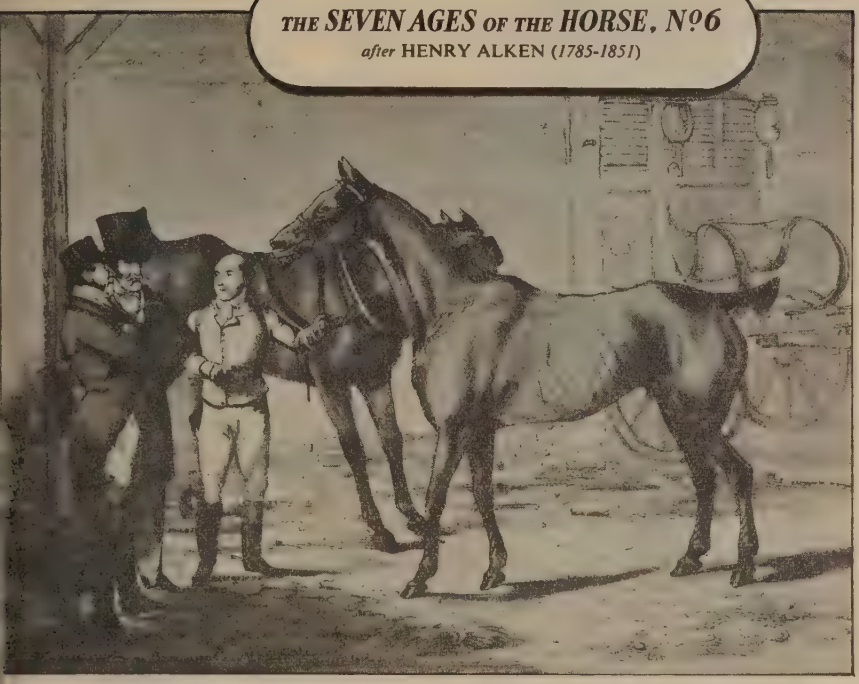

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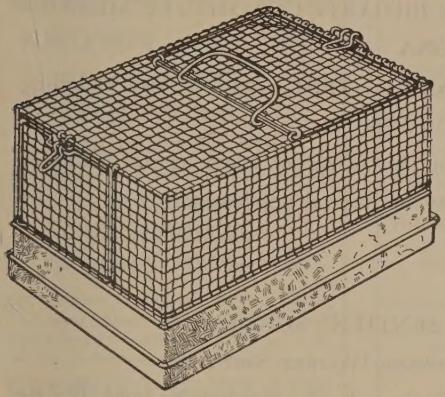

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